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DISMANTLING THE ABERRANT SIGNALING NETWORK IN CHRONIC LYMPHOCYTIC LEUKEMIA: PP2A AND SHP-1 AS PROMISING TARGETS FOR DRUG DISCOVERY

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CONTENTS

LIST OF ABBREVIATIONS

One and three-letter codes of amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
X		Any amino acid

Abbreviations of protein factors

SFK = Src Family Kinases

Lyn = Yamaguchi sarcoma viral related oncogene

APRIL = A Proliferation-Inducing Ligand

ATM = Ataxia Teleangectasia Mutated

BAD = Bcl-2 Associated Death promoter

BAFF = B-cell Activating Factor

Bcl-2 = B cell lymphoma gene 2

BCR= Recettore a cellule B

Bid =BH3-interacting domain death agonist

BLNK = B-cell Linker

BMSC = Cellule stromali midollari

BSA = Albumina di siero bovino

Btk = Bruton tirosina chinasi

CARD11 = Caspase Recruitment Domain 11

CD19 = Cluster of Differentiation 19

CD38 = Cluster of Differentiation 38

CD40L = Ligando di CD40

Csk = C-terminal Src kinase

CIP2A = Cancerous Inhibitor of PP2A

ERK = Extracellular signal Regulated Kinase

FADD = Fas-Associated Death Domain

FLIP= FLIce-Inhibitory Protein

GSK-3ß = Glycogen synthase kinase 3, ß isoform

HEAT = Huntington Elongation factor 3/pr65 A TOR

Hsp90 = Heat shock protein – 90 kDa

IL = Interleukin

ITAM = Immunoreceptor Tyrosine-based Activation Motif

ITIM = Immunoreceptor Tyrosine-based Motif Inhibitory Motif

I1PP2A = Inhibitor-1 of PP2A

I2PP2A = Inhibitor-2 of PP2A

CLL = Chronic Lymphocytic Leukemia

MAPK = Mitogen Activated Protein Kinase

NF-kB = Nuclear Factor κB

NK = Natural Killer

NLC = Nurse-Like cells

PARP = Poli-ADP-Ribosio Polimerasi

PI3K = Phosphoinositide-3-kinase

PKC = Protein kinase C

PLCγ2 = Phospholipase Cγ2

PP2A = Protein Phosphatase 2A

PTP = Protein Tyrosine Phosphatase

RTK = Receptor Tyrosine Kinase

SET = Suvar3-9 Enancer of zeste, Trithorax

SHP1/2 = SH2 domain-containing Tyrosine Phosphatase ½

SH1/2/3 = Src Homology Domain 1/2/3

SK1/2 = Sphingosine Kinase 1/2

Syk = Spleen tyrosine kinase

S1P = Sphingosine-1-phosphate

TCR = T Cell Receptor

TLR = Toll-Like Receptor

ZAP-70 = ζ-Associated Protein - 70 KDa

ABSTRACT (English)

Reversible protein phosphorylation is a fundamental post-translational modification by which virtually all cellular events are regulated, enabling cells to properly respond to intra- and extracellular cues. Protein kinases and protein phosphatases are the principal factors involved in this in this dynamic process and are placed at the different levels of cellular signalling, and, albeit traditionally considered as functionally opposed to one another, not rarely act in an interplay to finely orchestrate and appropriately drive signal transduction. The imbalance of their expression and function affects the cell life and fate, which frequently underlies the onset and progression of a plethora of diseases.

B cell chronic lymphocytic leukemia (CLL), the most common leukemia in the Western world, is no exception to this paradigm, many studies having highlighted a crucial role of kinases in the sustained signals from the signalosome downstream of the B cell receptor (BCR), and having led to the development of the promising second-line drugs, but also the blockade of a number of phosphatases underlying pro-survival and anti-apoptotic signals. In this regard, Protein Phosphatase 2A (PP2A) and the Src homology 2 domain-containing phosphatase 1 (SHP-1) exhibit a marked functional inhibition in this disease, which can be properly circumvented by a pharmacological approach, thereby inducing apoptosis of cancer cells. Nintedanib and MP-0766, a drug acting as an angiokinase inhibitor and a new fingolimod analogue devoid of immunosuppressive action, activating respectively SHP-1 and PP2A have enabled for the discovery of a signalling axis that when activated provokes massive cell death, and might provide a new paradigm for the treatment of CLL, which now endorses kinase inhibitors.

ABSTRACT (Italiano)

La fosforilazione proteica è una fondamentale modificazione post-traduzionale che regola virtualmente tutti i processi cellulari, permettendo alla cellula di rispondere a stimoli intra- ed extracellulari. Le protein chinasi e le protein fosfatasi sono i fattori principali coinvolti in questo processo dinamico e si localizzano a diversi livelli del signaling cellulare, e, sebbene tradizionalmente considerate opposte le une alle altre sotto il profilo funzionale, non raramente compartecipano per finemente modulare e opportunamente dirigere la trasduzione del segnale.

Uno squilibrio di espressione e/o funzione di questi fattori si riflette sulla vita e il destino della cellula, cosa che frequentemente è alla base dell'insorgenza nocnhé l'evoluzione di un gran numenro di patologie. La leucemia linfatica cronica a cellule B (B Chronic Lymphocytic Leukemia, CLL), la più comune leucemia in occidente, non fa eccezione a tale paradigma e, sebbene la ricerca per lo più si è concentrata sull'anomala attività di diverse protein chinasi con lo sviluppo di promettenti farmaci di seconda linea, sempre più di frequente viene confermata l'ipotesi che la sopravvivenza e la resistenza all'apoptosis delle cellule tumorali dipende anche dalla ridotta espressione o funzionalità delle protein fosfatasi. A questo riguardo, la protein fosfatasi 2A (Protein Phosphatase 2A, PP2A) e la fosfatasi 1 contenente domini Src homology 2 (Src Homology 2 domain-containing phosphatase 1 (SHP-1) in questa patologia si dimostrano funzionalmente inibiti, ma che, quando opportunamente attivate farmacologicamente, inducono morte delle cellule tumorali.

Nintedanib, un farmaco che agisce come inibitore "angiochinasico", e MP-0766, un nuovo analogo del fingolimod privo di azione immunosoppressiva, si sono dimostrati in grado di attivare SHP-1 e PP2A rispettivamente, permettendo inoltre di individuare un asse di signaling cellulare che provoca la morte di celle cellule leucemiche, potenzialmente rappresentando un nuovo paradigma per il trattamento della CLL, che ad oggi privilegia gli inibitori chinasici.

INTRODUCTION

CHRONIC LYMPHOCYTIC LEUKEMIA

Etiology, Diagnosis and Staging

It is the most common form of leukemia diagnosed in adults in Western countries, the median age at diagnosis being 72 years with an incidence of 4.2:100 000/year, which increases to >30:100 000/year in patients aged over 80 years (Eichhorst et al, 2015). As to the etiology of CLL, inherited genetic susceptibility for CLL seems to play a major role (Slager et al, 2013; Yang et al., 2015) although other possible caused including ionizing radiation (Linet et al., 2007) or the use of pesticides (Cocco et al., 2013) cannot be ruled out.

CLL is a dynamic disease with a highly heterogeneous clinical course varying from the stable, long-lasting indolent form to rapidly progressive disease and death (Chiorazzi et al., 2005; Stevenson and Caligaris-Cappio, 2004), one third remaining asymptomatic for years (Hallek et al., 2008), whereas others may experience an aggressive clinical course with a rapid increase in diseased cells in the blood and require very intensive treatment (Elphee, 2008).

The majority of CLL patients are asymptomatic, being accidentally diagnosed as a result of a routine blood test, which shows absolute lymphocytosis defined as more than 5000 cells/μL. 10% of patients present with symptoms consisting in unexplained fevers, unintentional body weight loss or night sweats, most of them having enlarged and palpable lymph nodes on examination, accompanied 20 to 50% of the cases by hepatosplenomegaly (Nabhan and Rosen, 2014). CLL cells then undergo further characterization by immunophenotyping, molecular genetics and cytogenetics to properly establish diagnosis and prognosis (see below).

Two clinical staging systems are used for predicting median survival, which classify patients on the basis of blood parameters and clinical signs such as hemoglobin concentration and thrombocyte count (Binet), lymphocyte/thrombocyte count and the presence or absence of lymphadenopathy/organomegaly (Rai) (Table 1) (Binet et al., 1981; Rai et al., 1975). The Binet staging system is widely used in Europe, whereas in the United States, the Rai system is more commonly employed.

Table 1. Rai and Binet Staging Systems

Rai

Stage	Risk	Clinical Features	Overall Survival, yrs
0	Low	Lymphocytosis in peripheral blood and bone marrow only	>10
I/II	Intermediate	Lymphadenopathy ± hepatosplenomegaly	7
III/IV	High	Anemia ± thrombocytopenia	<4

Binet

Stage	Risk	Clinical Features	Overall Survival, yrs
A	Low	<3 areas of lymphadenopathy	12
B	Intermediate	>3 areas of lymphadenopathy	7
C	High	Anemia, thrombocytopenia, or both	2-4

Cellular, molecular and functional features of CLL

Chronic Lymphocytic Leukemia (CLL) is a clonal lymphoproliferative disorder characterized by the slow accumulation of small, mature B lymphocytes in the peripheral blood, bone marrow and in the secondary lymphoid organs (spleen and lymph nodes). These leukemia cells express specific surface markers (so-called Cluster Differentiations, CD) such as CD19, CD5 and CD20, CD23, typicaly lacking CD22 and CD52 (Figure 1) with weak expression of surface

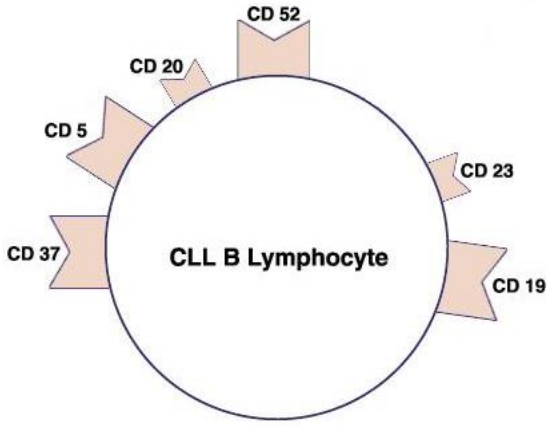

Figure 1. Typical surface markers of CLL B lymphocytes as described in http://www.campath.com/UnderstandingCLL/CellMarkers.html

immunoglobulins compared to normal B cells as established by flow cytometry and immunophenotyping of the peripheral blood (Nabhan and Rosen, 2014; Matutes

and Polliack, 2000; Eichorst et al., 2015). CLL cells undergo spontaneous apoptosis in vitro, from which they are protected when cultured in the presence of dendritic or nurse-like cells (NLCs), among others, NLCs being a subset of blood monocytes in CLL patients (Tsukada et al., 2002). This finding supports the hypothesis that the resistance to apoptosis of CLL cells, in addition of intrinsic defects, also depends on extrinsic factors, especially in the microenviroment of secondary lymphoid tissues from where these cells originate and mature (Herreros et al., 2008). Figure 2 shows the high complexity of such interactions (ten Hacken and Burger, 2016).

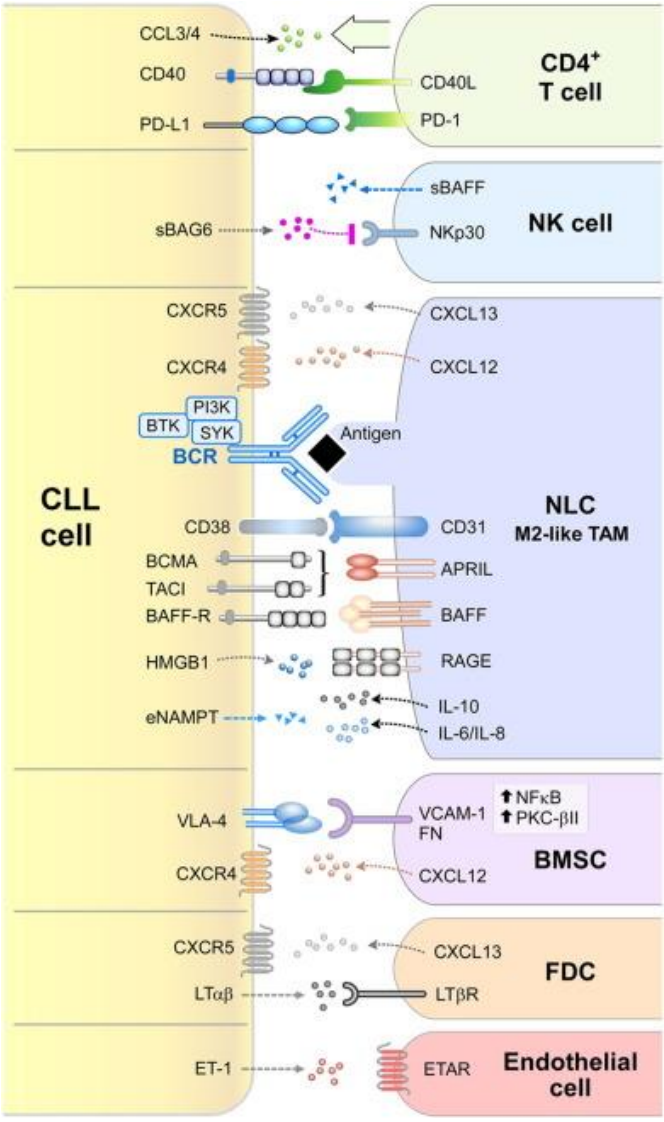

Figure 2. Various cellular and molecular components of the CLL microenvironment. Nurselike cells (NLCs); B Cell Receptor (BCR); Follicular Dendritic Cells (FDCs); Mesenchimal Stromal Cells (MSCs)

CLL cells in the blood smear are small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus with clumped chromatin. These cells may undergo partial breakdown during slide preparation because of the fragile nature of these cells ("Gumprecht shadows", Figure 3).

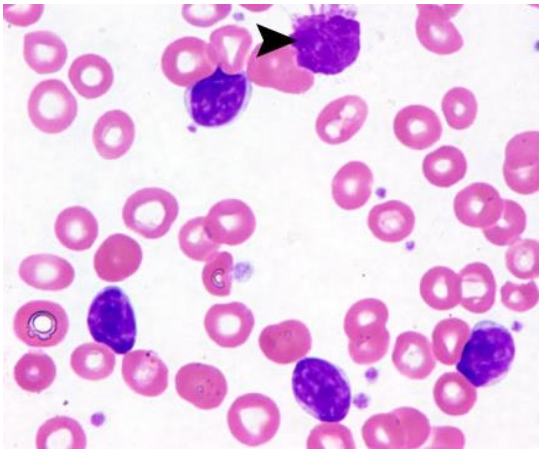

Figure 3. This smear shows the presence of four small neoplastic lymphocytes and one "Gumprecht shadow" (arrowhead).

Among the causes of the heterogeneity of the clinical course of the disease specific molecular alterations have been established, and namely the mutation of the genes coding for the Immunoglobulin Variable region Heavy chain (IgVH) and the level of expression of CD38 and Zeta chain Associated Protein kinase 70 (ZAP70) (Hamblin et al., 2007).

Mutational status of IgVH

Signal transduction in B cell is mediated by the B Cell Receptor (BCR), a transmembrane protein complex formed by an immunoglobulin (Ig) placed on the cell's outer surface connected to a network of kinases and phosphatases that tune and amplify its activation. The interaction with antigens are central to BCR's controlling B-cell fate and directing cell maturation, survival, anergy as well as the production of antibodies in plasma B cells (Reth and Nielsen, 2014; Dal Porto et al, 2004). The heavy and light chains of the Ig are the products of random rearrangement of immunoglobulin gene segments, which results in the highly diverse repertoire of Igs. On the basis of the degree of somatic mutation of the

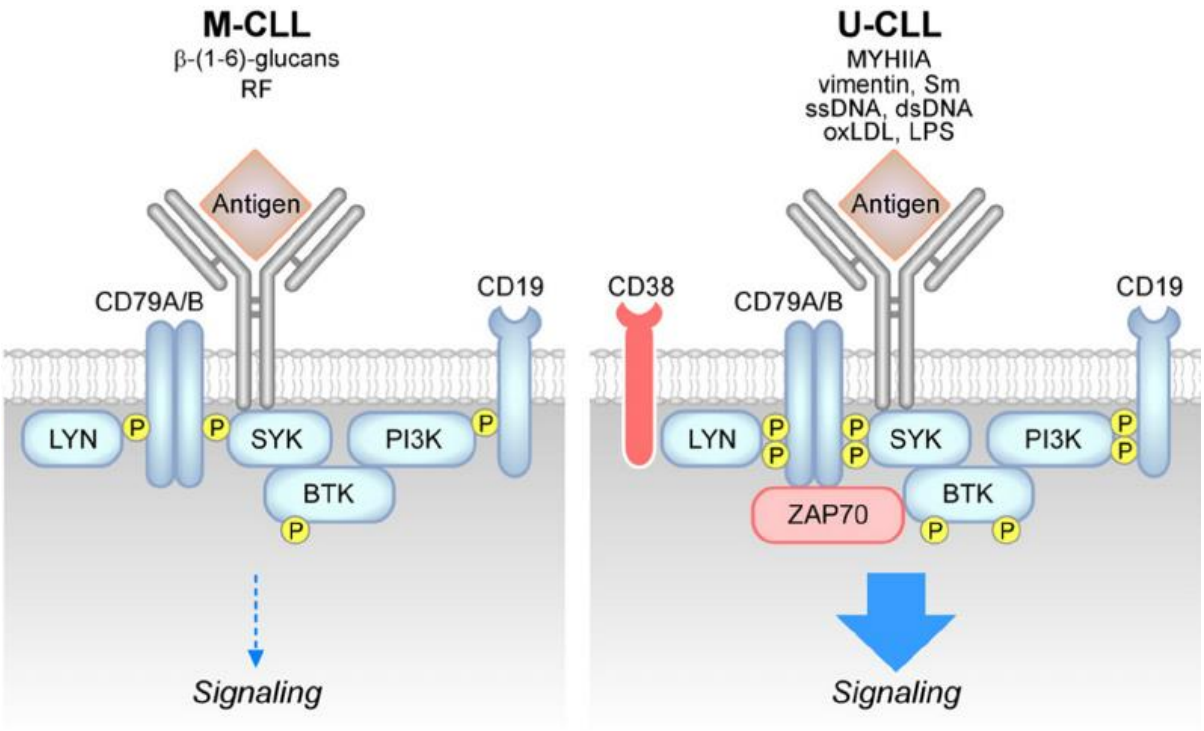

Figure 4. Differences between M-CLL and U-CLL signaling pathways (from ten Hacken and Burger, 2016).

IgVH genes, CLL patients can be classified into the 2 subgroups, unmutated CLL (U-CLL) or mutated CLL (M-CLL), depending on the strong or weak sequence homology with the sequence of the germ-line gene. U-CLL patients statistically have a poor prognosis with an aggressive course and often short survival, in contrast with those carrying IgVH mutations, who have a slow course and much longer life expectancy (Hamblin et al., 1999). The mutational status affects the BCR responsiveness to antigen stimulation, U-CLL patients expressing multireactive and non-selective BCRs binding a wide range of molecules present in the surrounding microenvironment, resulting in a sustained activation of BCR-dependent signals and a more aggressive disease (Burger and Chiorazzi, 2013). Figure 4 shows the organization of the "signalosome" on the inner leaflet of the plasma membrane of a CLL B cell functionally connected to the two mutational forms of BCR. U-CLL cells respond to a greater variety of antigens, the signals downstream of the BCR being reiforced by the contribution of the tyrosine kinase ZAP-70 and the multifunctional receptor CD38.

However, since the analysis of gene sequences underlying the status of IgVH mutation is laborious, reliable alternative markers are CD38 and ZAP-70, or the chemokines CCL3 and CCL4 .

ZAP70 and CD38

ZAP70 is a non-receptor tyrosine kinase normally expressed in T cells, Natural Killer (NK) cells, activated but not quiescent B cells. In fact, the majority of B lymphocytes expressing not this protein, but the tyrosine kinase named Syk. It is believed that its presence in B cells is linked to antigenic stimulation.

This protein is associated with the CD3 receptor of T lymphocytes, and is involved in signals downstream of the T Cell Receptor (T Cell Receptor) following antigen engagement (Wang et al., 2010). ZAP70 in CLL cells seems to play a similar function in the signals mediated by the BCR. Because ZAP70 is expressed in U-CLL cells, it is also used as a molecular marker to distinguish the mutational status of CLL cells. In fact, patients with ZAP70 being expressed in more than 20% of CLL cells, are more likely to experience an aggressive course of the disease. In addition, U-CLL cells expressing ZAP70 are more sensitive to chemokine (C-C motif) Ligand 19 (CCL19), CCL21 and chemokine (C-X-C motif) Ligand 12 (CXCL12), which stimulate migration and cell proliferation (Burger and Chiorazzi, 2013).

CD38 is a glycoprotein also known as cyclic ADP ribose hydrolase, which is found on the surface of many immune cells (Deaglio et al., 2006). It promotes cell proliferation by enhancing signals mediated by the BCR upon interaction with CD31, which is expressed on the surface of endothelial cells, among others (Deaglio et al., 2006). In addition to the proliferative response, emerging evidence indicates that CD31/CD38 interaction is also followed by migration and homing, suggesting a potential role for CD38 in directing CLL cells to specific microenvironments (Deaglio et al., 2010). Although CD38 was the first marker thought to be be closely related to the mutation status of IgVH genes, it was later proven that instead these two biological parameters are independent prognostic

factors (Hamblin et al., 2002). On the other hand, CD38 is also an important complementary prognostic marker, generally related to a more aggressive clinical course (Malavasi et al., 2011).

The CC chemokine family

The chemokines named CCL3 and CCL4, also called Macrophage Inflammatory Protein (MIP)-1α and -1β, respectively, are chemokines of the CC family, inducible in adaptive immune responses of cells of hematopoietic origin such as macrophages, B and T lymphocytes and dendritic cells. Plasma levels of CCL3 are indicative of an aggressive clinical course in CLL. Previous studies conducted on co-cultures of CLL cells with monocytes acting as NLCs have shown that the activation of the BCR-depending cascades induces a marked synthesis and secretion of CCL3 and CCL4, and, as expected, the use of inhibitors of players downstream of the BCR greatly reduces the levels of CCL3 and CCL4 (Burger et al, 2009). As to the actual contribution of CCL3 in the pathogenesis of the disease, it is hypothesized that the secretion of this chemokine serves to attract additional cells such as T lymphocytes, NLCs and stromal cells in the microenvironment, thus creating a favorable environment for survival of leukemia cell clones (Burger and Chiorazzi, 2013; de Weerdt et al., 2013).

Genetic lesions

There are several types of genetic lesions in CLL, which also represent prognostic markers of CLL (Rossi et al., 2013; Hallek, 2013). Among the genetic alterations, there are

- 13q14 deletion, present in more than 50% of patients, which also underlies the development of additional mutations in B cell clones;
- mutation of the NOTCH1 gene, coding for a transmembrane protein that acts as a ligand-dependent transcription factor and is involved in the processes of differentiation, proliferation and apoptosis through cell transcriptional

activation of other genes such as MYC, TP53 and molecules of the NF-kB pathway;

- mutations and deletions that cause degradation of BIRC3, gene implicated in the negative regulation of MAP3K14, which is the main activator of the non-canonical NF-kB signaling pathway;
- missense mutations of the SF3B1 gene, which is a basic component in the splicing process. The mutations in this gene are found in patients showing refractoriness to therapy (~ 15-20% of cases);
- mutations of MYD88, a factor participating in the response of Toll-like receptors, which causes an increased survival due to cell to constitutively activated signal pathway mediated by NF-kB;
- deletions, or somatic mutations such as missense mutations in exons 5-8 of the TP53 gene will cause the inactivation of the protein product, which is a nuclear factor involved in the regulation of apoptotic response upon DNA damage;
- mutations of the Ataxia Telangiectasia Mutated (ATM) gene, which encodes a protein kinase that phosphorylates and activates p53 in response to damage to the DNA double helix. This injury causes the inactivation of ATM in only 30% of the cases.

  The lesions of the ATM and TP53 genes confer resistance to chemotherapy to CLL cells, since both genes are involved in the process of apoptosis. Besides, these deletions are associated with patients with U-CLL, who have a poor prognosis.
- Trisomy 12 has been shown in 25% of U-CLL patients. This chromosome harbors the murine double minute 2 (MDM-2) gene, the product of which is an important regulator of the tumor suppressor gene TP53. Its overexpression leads to functional inactivation of the p53.
- Deletions of chromosome 6, present in about 6% of cases, causing minor events related to intermediate prognosis.

Signaling in CLL cells: overview

Reversible protein phosphorylation is a fundamental post-translational modification by which virtually all cellular events are regulated, enabling cells to properly respond to intra- and extracellular cues. The crucial players involved in this dynamic process are protein kinases and protein phosphatases, which are placed at the different levels of cellular signalling, and, albeit traditionally considered as functionally opposed to one another, not rarely act in an interplay to finely orchestrate and appropriately drive signal transduction (Johnson, 2009). The significance of both classes of enzymes in the cell life and fate is mirrored by the effects of their dysregulation, whether related to altered expression or activity, which frequently underlies the onset and progression of a plethora of diseases (Lahiry et al., 2010; Julien et al., 2011). CLL is no exception to this paradigm, a high level of intracellular phosphorylation being mediated by the abnormal activity of several kinases downstream of the B cell receptor (BCR), such as Lyn (Contri et al., 2005), Syk (Gobessi et al., 2009), PKC (Ringshausen et al., 2002), phosphoinositide 3-kinase (PI3K) (Plate, 2004), mitogen-activated protein kinase (MAPK) p38 (Sainz-Perez et al., 2006), resulting in a ligand-independent BCR signaling, also defined tonic signaling (Monroe, 2006). The peculiar structural and functional features of the BCR in CLL, which are related to the presence or absence of somatic mutations of the IgVH genes, often associated with atypical expression of ZAP-70, dysregulate the downstream signaling pathways, directly contributing to the onset and progression of the disease (Lanham et al., 2003; Rassenti et al., 2004). Importantly, this has also led to new promising therapeutic approaches that interfere with aberrant kinase activities and are now employed in clinical practice (Figure 2, also see below). Whilst the role of dysrgulated kinase activities has been ascertained, accumulating evidence is consistent with the hypothesis that the abnormal signal transduction is also the result of the lack of a proper counterbalance mediated by a number of phosphatases, whose expression or activity is altered in CLL cells. For instance, PTEN (Zou et al., 2015), PTPROt

(Motiwala et al., 2007), PHLPP1 (Suljagic et al., 2010) and SHIP1 (Cui et al., 2014) exhibit significantly decreased expression, leaving tonic pro-survival signalling intact, whereas PTPN22, which acts as a positive regulator of anti-apoptotic signals by hampering the negative regulation of BCR-dependent signalling pathways, is overexpressed (Negro et al., 2012). By contrast, Protein Phosphatase 2A (PP2A) (Perrotti and Neviani, 2008) and the Src homology 2 domain-containing

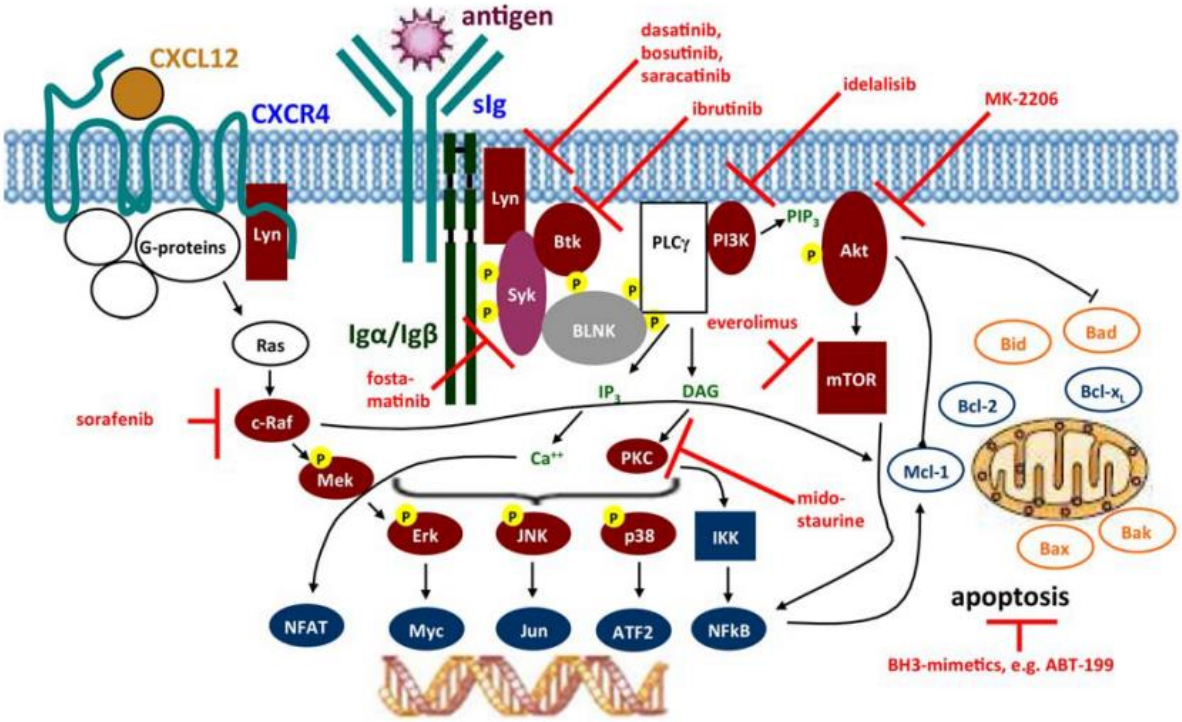

Figure 5. Factors taking part in aberrant BCR-dependent signaling and targeted by specific agents, which represent a new strategy in the treatment of CLL (from Hallek, 2013).

phosphatase 1 **(**SHP-1) (Tsui et al., 2006) are expressed in CLL at levels comparable to normal B cells, but are functionally dysregulated by a variety of mechanisms, which include phosphorylation, interaction with cellular partners and location and are chiefly mediated by the Src Family Kinase (SFK) Lyn (Zonta et al., 2015; Tibaldi et al., 2011). In this scenario, not only this imbalance, but also a direct connection between kinases and phosphatases, with vicious cycles that establish aberrant signalling axes (Zonta et al., 2015) might be the new perspective for the identification of novel drug targets.

Current pharmacologic treatments

Patients with early-stage (Binet stage A and B without active disease; Rai 0, I and II without active disease) do not experience a survival advantage if they undergo chemotherapy (Dighiero et al., 1998), the standard approach being a watch-and-wait strategy. In contrast, treatment should only be initiated in patients with symptomatic, active disease (Binet stage A and B with active disease or Binet stage C; Rai 0 – II with active disease or Rai III – IV). Standard front-line treatment in physically fit patients consists of fludarabine, cyclophosphamide and rituximab (FCR), which has been shown to improve overall survival (Hallek et al., 2010), though being associated with severe infections in fit elderly patients when compared to bendamustine (a purine analog like fludarabine) and rituximab. Other purine analogs such as cladribine (Robak et al., 2010) or pentostatin (Kay et al., 2007) have shown similar activity. In older patients with relevant co-morbidity and without TP53 deletion/mutation, progression-free survival has been observed by combining chlorambucil and an anti-CD20 antibody (rituximab, ofatumumab or obinutuzumab) when compared with monotherapy and is therefore the standard approach (Goede et al., 2014; Hillmen et al., 2013).

Patients with TP53 deletion/mutation should be treated with novel agents (the Bruton's tyrosine kinase inhibitor ibrutinib or the combination of the PI3K inhibitor idelalisib with rituximab), since they have a poor prognosis even after FCR therapy (Hallek et al., 2010), either as a front line-therapy or an approach to treat relapses. An allogeneic hematopoietic stem-cell transplantation may be taken into consideration in fit patients responding to kinase inhibitors (Dreger et al., 2014).

As to relapse and refractory disease, asymptomatic CLL patients should take no therapy for a long period of time. First-line treatment may be repeated if the relapse or progression occurs at least 24–26 months after chemoimmunotherapy and if TP53 deletion/mutation was excluded, whereas relapse occurring within 24–36 months after chemoimmunotherapy, or the lack of responsiveness to any therapy requires a change in the therapeutic (Eichorst et al., 2015). Treatment options include:

- BCL2 antagonists, such as ABT-199, alone or in combination within a clinical study (Roberts et al., 2016)
- Bruton's tyrosine kinase inhibitor ibrutinib (Byrd et al., 2014)
- PI3K inhibitor idelalisib in combination with rituximab (Furman et al., 2014).

The drugs used in clinical practice as described above are only few of the potential agents that might be approved for CLL treatment, especially if we consider how complicated the network of signals generated by the many factors just beneath the plasma membrane of a CLL cell is (Figure 5). In this regard, as confirmed by the current second-line approach, the most interesting and promising molecules are the kinase inhibitors.

Kinase inhibitors in the treatment of CLL

As described above, there are kinase inhibitors that have been approved as second-line therapy in CLL patients. A few of them are described below and include the already mentioned ibrutinib and idelalisib, which are the most effective and currently utilized in the treatment of this disease. (also see Figure 5, where various agents targeting components of the BCR signaling network are shown, although not yet approved for therapy or already shown scarcely efficacious if administered alone).

Dasatinib. It is a second-line drug used for the treatment of chronic myeloid leukemia (CML) (Keating, 2017). It can also inhibit Src Family Kinases (SFKs), such as Lyn, and Btk. Its application should be considered not as a single-agent therapy in CLL but in combination to sensitize tumor cells to fludarabine-mediated apoptosis (Aguillon et al., 2007).

Ibrutinib. Irreversible inhibitor of Btk, it barely induces apoptosis of CLL in vitro. Ibrutinib can counteract the protective effect of CD40L, BAFF, TNF-α, IL-6, and IL-4 (Herman et al., 2011), also preventing the adhesion induced by anti-IgM

antibodies, and the adhesion and migration of cells in response to chemokines (de Rooij et al., 2012). It has been to be able to induce the release of CLL cells from lymph nodes and bone marrow, considering the short rise in the lymphocyte count in the peripheral blood occurring following therapy, where they are no longer protected by the microenvironment, becoming prone to apoptosis (Chang et al., 2013). Ibrutinib has been tested not only as a single agent but also in combination therapy (Byrd et al., 2014).

Fostamatinib. It is a prodrug, its active metabolite being R406 and acting as a Syk inhibitors. It has also been studied for the treatment of autoimmune diseases such rheumatoid arthritis. In vitro fostamatinib, by inhibiting Syk, jeopardizes the viability of CLL cells, which cannot be rescued even when stimulated with anti-IgM antibodies or placed in co-cultures with the NLCs. It exhibits synergy with fludarabine (Herman et al., 2013).

Idelalisib. Also termed GS-1101 or CAL-101, it selectively inhibits the PI3Kδ p110δ isoform in vitro, thereby inducing apoptosis of CLL, and reducing the migration by blocking the signals generated by CXCL12 and CXCL13. It also shows fewer cytotoxic effects on NK cells when compared with non-selective inhibitors of PI3K (α, β, δ, γ) (de Weerdt et al., 2013).

Lyn and its substrates: anti-apoptotic strategy of CLL cells

One approach aimed at the discovery of new therapeutic targets in CLL is to explore the intracellular pathways responsible for modulating the proliferation and/or apoptotic rate of CLL cells. The extracellular cues that are sensed by the BCR in concert with other co-receptors need to be translated by intracellular switches, most of which are protein kinases, as described above. In the last ten years, the Src Family Kinase (SFK) Lyn has emerged as a factor central to sustaining the cancer phenotype of CLL cells and orchestrating the aberrant signaling network downstream of the BCR. In fact, it is the first protein kinase to

transduce the signal from BCR by phosphorylating the Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) placed on Igα / Igβ chains of the BCR, thus triggering the recruitment of other kinases and adapter molecules downstream, such as Syk, PLCγ2, PI3K, Btk, Vav and BLNK, which form the "signalosome". This multiprotein complex propagates the signals downstream and activates the signaling pathways that support proliferation, and/or survival through the activation of anti-apoptotic mechanisms (Contri et al., 2005; Tibaldi et al, 2011). In addition to a "positive" role, Lyn has also found to exert a "negative" action, by phosphorylating the inner tail of CLL surface marker CD5 and thus recruiting the tyrosine phosphatase SHP-1, which contributes to the anti-apoptotic signals by dephosphorylating specific targets such as Vav1 (Tibaldi et al, 2011; also see below). Yet, Lyn has been shown to be located not only at the plasma membrane, as expected, but also in the cytosol as a member of a multiprotein complex associated with HSP90, where it is maintained constitutively active, thus explaining the high level of tyrosine phosphorylation observed in this compartment of CLL cells (Trentin et al., 2008). The discovery illustrated above also spurred new research into the nature of possible Lyn's cytosolic substrates with a view to extend the repertoire of new therapeutic targets of this disease, especially in the light of the disappointing results in managing Lyn-targeting drugs, such as dasatinib (McCaig et al, 2011; Kater et al., 2014). In this regard, the first evidence of how Lyn's dysregulated expression, activity, and localization mediate resistance to apoptosis in CLL by affecting the phosphorylation status of a specific substrate in the cytosol emerged through the identification of procaspase-8. In fact, the phosphorylation at Y380 was shown to result in the dimerization of the caspase zymogen, blocking the so-called extrinsic pathway of apoptosis, whereas the use of SFK inhibitors abolished the phosphorylation of this initiator caspase, inducing its activity and initiating the apoptotic cascade (Zonta et al., 2014). Interestingly, by using both dasatinib and other SFK inhibitors, it was observed that not only tyrosine phosphorylation decreased, thus bringing about cell death by a caspase-dependent mechanism, but also caused a drastic drop of the phosphorylation of

specific serine residues of protein kinase that are central to cell survival, such as Akt (Ser473) and the substrate thereof GSK-3 (Ser9). This evidence suggested that a yet-to-identify protein phosphatase, which reasonably might be thought to be downregulated by Lyn, was involved in the dephosphorylation of these proteins. Interestingly, SFK inhibitors also induced the dephosphorylation of the inhibitory site of the tyrosine phosphatase SHP-1, S591, raising the question whether SHP-1 might be implicated in the control of apoptosis and hence might represent a potential pharmacological target (see below for details). Importantly, the fact that the dephosphorylation of these factors was counteracted by okadaic acid at concentrations within the nanomolar range led to the reasonable hypothesis that the protein phosphatase responsible for these events was Protein Phosphatase 2A (PP2A) (Zonta et al., 2015). This was also corroborated by the observation that two mechanisms of inhibition of the phosphatase itsef occurred, namely a) PP2A was phosphorylated at Tyr307 of its catalytic subunit (PP2Ac), an inhibitory site targeted by tyrosine kinase (Barisic et al., 2010), plausibly Lyn in CLL, considering its overactivity and that b) the oncoprotein SET/I2PP2A, which is known to act acts as PP2A endogenous inhibitor (Christensen et a., 2011), was tightly bound to PP2A, this interaction being strengthened by the phosphorylation at Tyr307 (Zonta et al., 2015).

For a better understanding of the role of the inhibition of phosphatases in CLL, the structure, regulation and function of PP2A and SHP-1 illustrated below.

PROTEIN PHOSPHATASE 2A: MASTER REGULATOR OF VIRTUALLY ALL CELLULAR PROCESSES

Protein Phosphatase 2A (PP2A) constitutes a major family of serine/threonine phosphatases, being ubiquitously expressed and highly conserved among the species (mostly eukariotes) (Sangodkar et al., 2016). It is also abundantly expressed, accounting for up to 1% of total cellular protein in some tissues (Shi, 2009) and contributing, along with Protein Phosphatase 1, to over 90% of all serine/threonine phosphatase activities in most tissues and cells (Eichhorn et al., 2009). PP2A regulates a great variety of cellular functions including metabolism, cell cycle, DNA replication, transcription and translation, proliferation, and apoptosis (Seshacharyulu et al., 2013). Its role as a tumor suppressor was first suggested when okadaic acid (OA), an already known carcinogen, was shown to selectively inhibit PP2A (Nagao et al., 1989). Additional evidence that its inhibition might lead to malignant transformaton emerged from studies on the oncogenic simian vacuolating virus 40 (SV40) small T-antigen, probably capable of disrupting the heterotrimeric structure of PP2A (Mumby, 1995). Furthermore, different components of the PP2A holoenzyme were shown to be mutated in various forms of cancer, *e.g.* CML, acute myeloid leukemia (AML) or lung cancer; on the other hand, the restoration of PP2A activity has been shown to induce cell death in cancer cells (both *in vitro* and *in vivo*) and counteract uncontrolled proliferation (Eichhorn, 2009; Oaks and Ogretmen, 2015).

PP2A: structure

Active PP2A holoenzyme is composed of three main subunits,

- $PP2A_C$, the catalytic subunit (in green in figure 6)
- $PP2A_A$, the structural subunit (in red)
- $PP2A_B$, the regulatory subunit (in blue).

The core of the enzyme is made up of $PP2A_C$ and $PP2A_A$, and can exist as a dimer (one third of the total cellular PP2A) or associated with one of the different types of B subunits. The numerous genes and isoforms of PP2A subunits result in a large

heterogeneity of PP2A holoenzymes (Ramaswamy et al., 2015), with nearly 100 possible PP2A holoenzymes that can be found in the cell (Haesen et al., 2014).

$PP2A_C$: the catalytic subunit

$PP2A_C$ is responsible for the catalytic activity of the enzyme. $PP2A_C$ is a 36 kDa subunit, with two isoforms, Cα and Cβ, having 97% identity in their primary sequence (Seshacharyulu et al., 2013; Janssens and Goris, 2001). These two isoforms are ubiquitously expressed (with peaks in brain and the heart), being $PP2A_C$α predominant at the plasma membrane and $PP2A_C$β in the cytoplasm and nucleus; their expression levels are different, since the α isoform is 10 times more abundant than β. (Janssens and Goris, 2001). As explained below, this subunit undergoes a variety of post-translational modifications and interacts with protein partners, which all affect the enzyme activity.

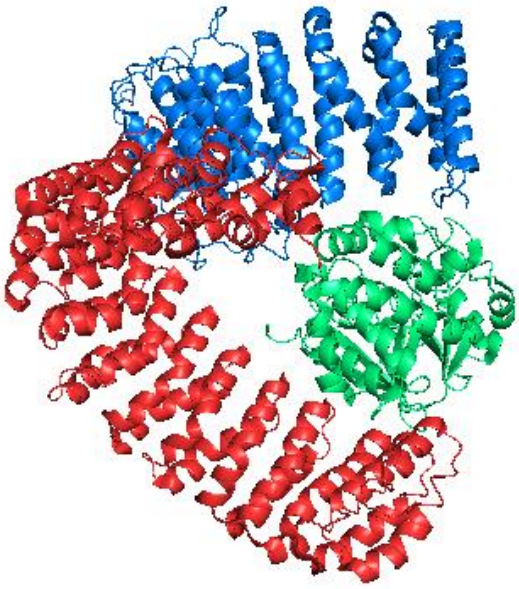

Figure 6. PP2A heterotrimer; modified from PDB ID: 2NYM.

$PP2A_A$: the structural subunit

$PP2A_A$, also known as PR65 or structural subunit, is the structural docking platform for the subunits B and C. The structure of $PP2A_A$ is composed of 15 tandem repeats of 39 amino acids termed HEAT (huntingtin/elongation/A subunit/TOR) motif

(Janssens and Goris et al., 2001). As already seen for $PP2A_C$, also $PP2A_A$ is encoded by two genes which lead to the production of two protein isoforms, α and β, which share around 87% of sequence identity and a molecular weight of 65 kDa. 90% of PP2A holoenzymes contain the α isoform and 10% the β, likely due to different roles in the cell.

$PP2A_B$: the regulatory subunit

$PP2A_B$ represent the most heterogeneous group of PP2A subunits, which totally lack structural similarities (Eichhorn et al., 2009; Janssens and Goris, 2001). The presence of this subunit is extremely important in that it is related to tissue expression, interaction with protein partners and substrate specificity. They can be classified into four distinct families.

B family (PR55)

This first family comprises four distinct subunits of 55 kDa each (called α, β, γ, δ,). α and δ are mostly ubiquitous in comparable amounts, β and γ are highly enriched in the brain. One of the most interesting features of this family is the presence of five degenerate Trp/Asp-40 (or WD-40) repeats, *i.e.* sequences composed of a partially conserved 40 amino acid sequence and ending with Trp-Asp. These portions are thought to mediate protein-protein interaction, for example the one with TGF- β receptors (Janssens and Goris, 2001).

B' family (PR61)

These subunits have a molecular weight of 61 kDa and are encoded by five genes which, due to their splice variants, lead to the production of seven subunits. They show a peculiar and precise distribution in the cell: α, β, and ε are mostly found in the cytoplasm, γ1, γ2 and γ3 are mostly found in the nucleus, while *B'*δ is expressed in both compartments the nucleus and the cytoplasm (Eichhorn et al., 2009). All the PR61s are structurally similar in the central part (80% of sequence identity), differing at N- and C- termini. It has been shown that this type of subunits exposes

a highly acidic surface, which is believed to be responsible of further protein recruitment (Eichhorn et al., 2009).

B'' family (PR72)

Little is known about this family, even as to the number of the members, though it is thought that there are four to five (Janssens and Goris, 2001). While PR130 is ubiquitous, PR72 is expressed exclusively in the heart and skeletal muscle, (with highest levels in heart and muscle), PR59 in testis, kidney, liver, brain, heart and lung; PR48 and G5PR are still matter of discussion (Seshacharyulu et al., 2013; Janssens and Goris, 2001)

B''' family

These subunits have been shown to share a conserved structure with WD-40 repeats that are typical for B family. They are involved in Ca^{2+}-mediated signaling (Janssens and Goris, 2001).

PP2A: function

Considering the potential impressive number of combinations of its subunits, it is reasonable to assume that PP2A has a considerable number of functions. As a master regulator of signal transduction, PP2A directly influences cell proliferation, differentiation, adhesion, migration, metabolism and, most of all, survival (Haesen et al., 2014). Figure 7 shows only a few of the substrates regulated by PP2A (Ciccone et al., 2015).

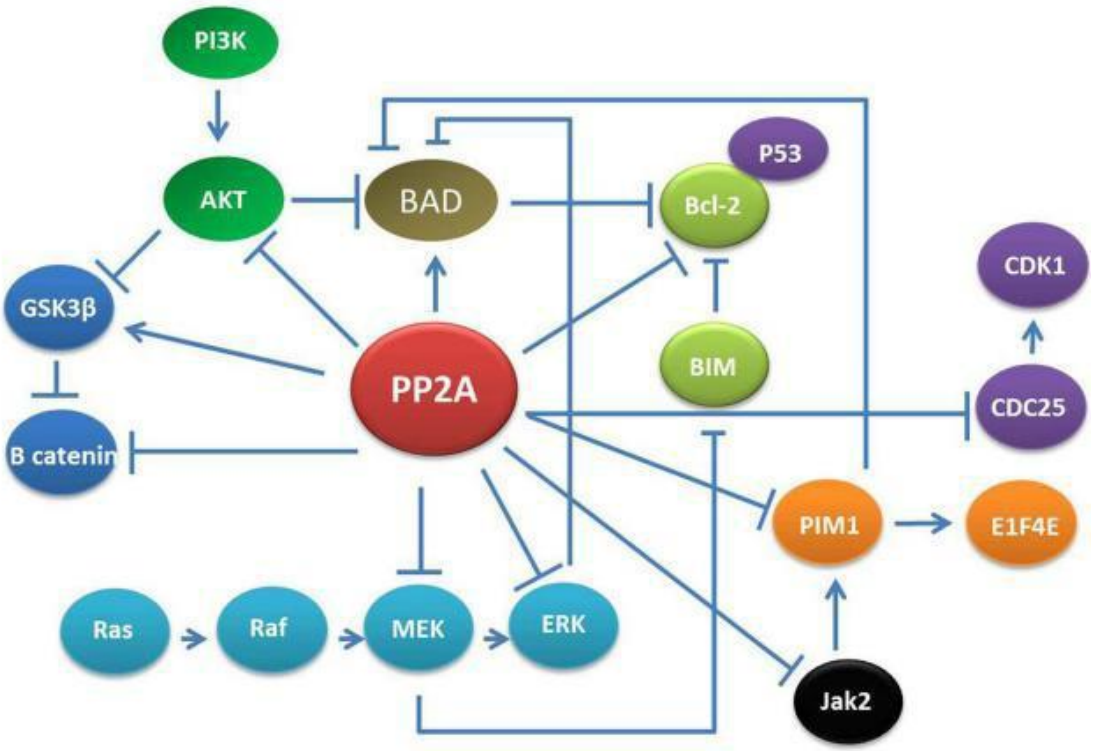

Figure 7. PP2A is involved in various cellular signals ranging from growth, survival to the cell cycle, to name a few. PP2A down-regulation may affect multiple pathways resulting in alteration of apoptosis, cell growth, proliferation, and differentiation in adult cells (from Ciccone et al., 2015).

PP2A as a tumor suppressor

PP2A is considered a tumor suppressor. First evidence was the pro-oncogenic effect of okadaic acid (OA), a specific PP2A inhibitor (Bialojan and Takai, 1988). However, stronger evidence arose from studies on eukaryotic cells, which showed how the imbalance of the expression of one or more subunits caused severe misregulation in cell life; for instance,

- overexpression of $PP2A_A\alpha$, which leads to the sequestration of the catalytic subunit, subsequent inefficient dephosphorylation and activation of myosin light chain kinase followed by inhibition of cytokinesis and the formation of bi- or multi-nucleated cells (Wera et al., 1995);
- truncation of PR61 leads to radio-resistance and metastasis, PP2A being involved in the regulation of paxillin and Mdm2 (inhibitor of the p53 tumor suppressor). The decreased dephosphorylation of paxillin (adaptor molecule

involved in cell migration), enhances cell motility promoting metastasis (Ito et al., 2000);

- mutations of $PP2A_A$ have been demonstrated to be present in a variety of human cancers, specially lung tumors, lung-derived tumors and colon tumors (Wang et al, 1998), while mutations of PPP2R1B gene are involved in the onset of almost all types of colorectal cancers (Takagi et al., 2000);
- deletion of PR55 in eukaryotes leads the activation of MAPK signaling, responsible for cell overgrowth (Silverstein et al., 2002); the deletion of either $PP2A_A$ or $PP2A_C$ provokes the disappearance of all PP2A subunits; ablation of total PP2A by using dsRNA against either A or C subunit enhances ERK activation (Silverstein et al., 2002).

This information indicates that a broad comprehension of the properties and the regulation of this enzyme could lead to a new approach in new strategies of cancer treatment.

PP2A and apoptosis

Apoptosis – or programmed cell death - is a crucial event for cellular turnover; lack or excessive apoptotic signals is a common hallmark of diseases such as neurodegenerative disorders, ischemia, autoimmune disorders and cancer. The capability of modulating cell life and death is an extremely desirable pharmaceutical aim because of its huge therapeutic potential (Elmore, 2007).

For instance, β-catenin is a central component of the Wingless-type MMTV integration site (Wnt)/β-catenin pathway. β-catenin activates growth and anti-apoptotic mechanisms due to the activation of c-MYC genes. PP2A inactivates this pathway and blocks these pro-survival signals (Perrotti and Neviani, 2013).

PP2A is also involved in one of the main apoptosis-modulating signaling cascades: the phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT) pathway, which supports cell survival, proliferation, cell cycle progression, proliferation,

angiogenesis, and self-renewal of stem cells (Yoon et al., 2010). PP2A suppresses this pathway by directly dephosphorylating and inactivating Akt and by suppressing Akt-activation signals (Ciccone et al., 2015). Akt exerts an anti-apoptotic effect by inhibiting the release of cytochrome C from mitochondria through phosphorylation of BAD (Bcl-2-associated death promoter) as well as pro-caspase-9 (Perrotti and Neviani, 2013). The same pathway partly also involves the inactivation of Bcl-2 (B-cell lymphoma 2), an anti-apoptotic factor, and the activation of BAD and Bim (Bcl-2-like protein 11).

PP2A: regulation

Considering the fundamental function of PP2A, it must be strictly regulated. Moreover, given the many possible isoform combinations, the modes of regulation of PP2A are quite heterogeneous, and can be classified mainly into post-translational modifications and interaction-mediated modulation.

Post-translational modifications

The main post-translational events that can affect PP2A activity are phosphorylation and methylation, both occurring on the catalytic subunit.

The phosphorylation of Thr304 provides steric hindrance hampering the recruitment of other subunits, while that of Tyr307 prevents methylation of the C-terminus, which occurs at Leu309 and is mediated by Leu-carboxyl methyltransferase of type I (LCMT1) (Perrotti and Neviani, 2013). The addition of a methyl residue to the Leu leads to the recruitment of B subunits other than than B' and B'', which instead prefer non-methylated $PP2A_C$ (Perrotti and Neviani, 2008).

Physiological modulators: cellular inhibitors

I1PP2A

The inhibitor-1 of PP2A (I1PP2A), also known as acidic leucine-rich nuclear phosphoprotein 32 A (ANP32A), is a 32-kDa sphingosine/dimethylsphingosine-sensitive PP2A inhibitor (Habrukowich et al., 2010). It also functions as part of the

histone acetyltransferase regulatory INHAT complex in the nucleus, which also contain, as will be discussed below, another PP2A inhibitor, the oncoprotein named I2PP2A/SET) (Seo et al., 2001). It interacts with the catalytic subunit of PP2A ($PP2A_C$) alone or when the latter is complexed with $PP2A_A$. This binding is weakened by tyrosine phosphorylation of ANP32A, with consequent elevation of PP2A activity (Yu et al., 2004).

I2PP2A (SET)

The inhibitor-2 of PP2A (I2PP2A), also known as SET (Suvar3-9, enhancer of zeste, trithorax, hereafter referred to as SET) is a 28-kDa protein (Figure 8) that is able to interact and inhibit $PP2A_C$. Its function is also regulated by the phosphorylation of a number of serine residues including Ser9, which interferes with the nuclear localization signal and accounts for its retention within the cytoplasm (Yu et al., 2013). The constitutive phosphorylation that results from the SET's inhibitory action involves PP2A substrates including Akt, PTEN, ERK1/2, Mcl-1, c-Myc, c-jun, and pRb (Haesen et al, 2014), which can then dysregulate a number of signaling pathways giving rise to diseased conditions such as cancer above all (Ruvolo et al., 2016).

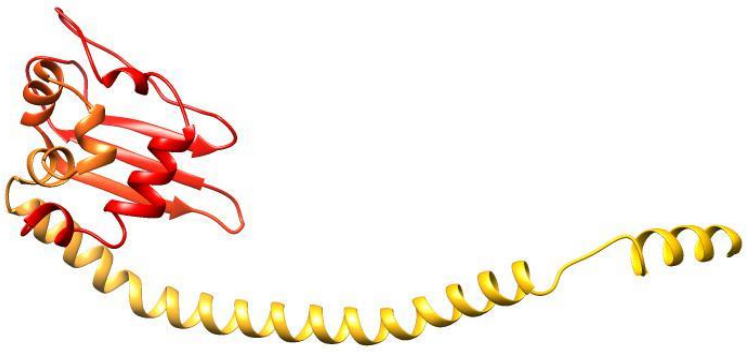

Figure 6. Crystal structure of SET protein, monomer (modified from PDB ID 2E50).

CIP2A

CIP2A (cancerous inhibitor of PP2A) is a 90-kDa oncoprotein which was found to be tightly bound to $PP2A_A$ rather than the catalytiuc subunit of the heterotrimer, in lymphocytes from leukemia patients. Scarcely expressed under physiological conditions, its overexpression is associated to the development of a great number of neoplasias, although a lack of expression alters spermatogenesis. Several oncogenic transcription factors including Ets, Myc and E2F increase the expression of CIP2A, which in turn represents a marker of cancer aggressiveness and poor prognosis (Khanna et al., 2013). CIP2A ultimately correlates with the phoosphorylation and stability of c-Myc, enhanced Akt-mediated signaling, and dysregulation of protein kinases implicated in the regulation of the cell cycle such as NEK2 and PLk1. In addition, the activation of the mTOR pathway has been observed (Haesen et al,, 2014).

Physiological modulators: cellular activators

Ceramides

Ceramides are sphingolipids composed of a fatty acid bound to the amine moiety of sphingosine (Figure 9). These are sphingolipid metabolite and structural components of the cell membrane with potent signaling properties (Janssens and

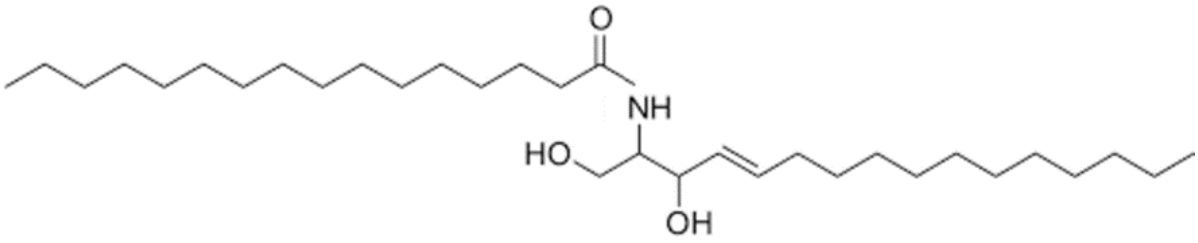

Figure 9. Ceramide

Rebollo, 2012) as well as characteristic activators of PP2A. Ceramides has been shown to activate or restore the pro-apoptotic activity of PP2A, but the mechanism by which this occurs is poorly understood. It has been reasonably hypothesized that this sphingolipid binds to SET, thereby interfering with the formation of a SET/PP2A complex and preventing the inhibition of the phosphatase activity (Saddoughi et al., 2013). In this regard, ceramide can induce apoptosis in prostate-

cancer cells by suppressing the Akt signaling pathway, which depends on the reactivation of PP2A (Perrotti and Neviani, 2013). As will be discussed later, an analogue of spingosine, the ceramide precursor, named fingolimod (FTY720, Gilenya®, Figure 6), a drug approved for oral therapy in multiple sclerosis, has proved effective in activating PP2A by interfering with the interaction with SET (Saddoughi et al., 2013).

Non-physiological inhibitors

Okadaic acid (OA)

OA is the prototypical PP2A inhibitor, a toxin produced by dinoflagellates (Reguera et al., 2014), and that reversibly binds within the catalytic pocket of $PP2A_C$. At concentrations within the nanomolar range, it proves highly selective towards PP2A, which has allowed for the exploration of PP2A as a tumor suppressor.

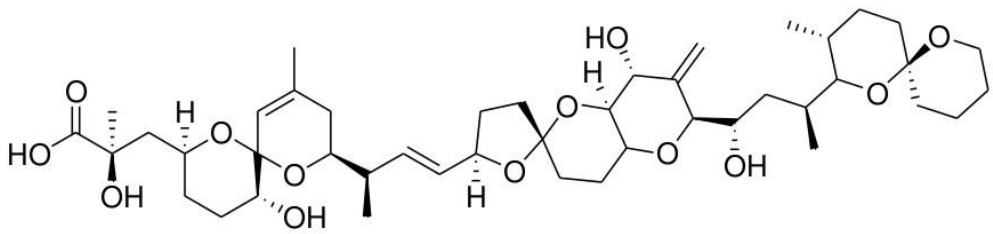

Figure 10. Okadaic acid

Forskolin

Forskolin is a diterpene capable of raising the intracellular levels of cAMP, but also to activate PP2A, especially in the form of 1,9-dideossi forskolin, which lacks the ability to activate adenylyl cyclase. In CML, a marked apoptosis has been observed following reactivation of PP2A by forskolin (Perrotti e Neviani, 2013).

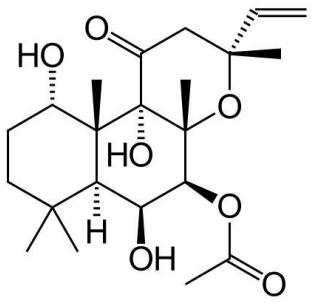

Figure 11. Forkolin

Fingolimod (FTY720)

As mentioned above, fingolimod (2-Amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol, hereafter FTY720, Figure 12) is the first oral drug approved in the treatment of the relapsing-remitting form of multiple sclerosis and marketed as Gilenya® by Novartis. First identified within a library of compounds synthesized in an effort to develop derivatives of myriocin, a metabolite of the fungus *Isaria sinclairii* sharing close structural similarity to sphingosine and displaying a potent

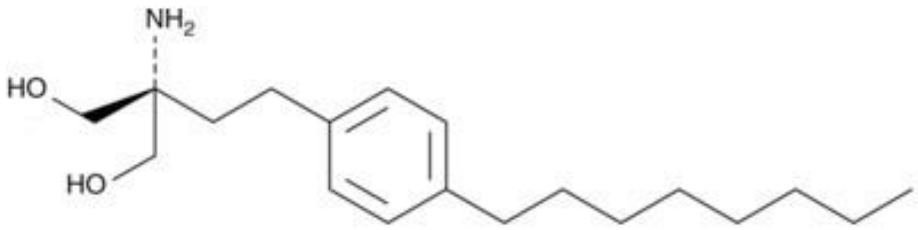

Figure 12. Chemical structure of fingolimod (FTY720)

immunosuppressive action by inhibiting serine palmitoyltransferase, a crucial enzyme in sphingolipid metabolism (Miyake et al., 1995), FTY720 sparked considerable interest in the area of organ transplantation in that a number of preclinical studies using animal models demonstrated the efficacy of FTY720, alone or in combination with cyclosporine, in prolonging solid organ allograft survival. (Brinkmann and Lynch, 2002; Budde et al, 2006). Importantly, FTY720 is considered a prodrug, because it must first be phosphorylated by sphingosine kinase 2 (SK-2) (Pelletier and Hafler, 2012; Pitman et al., 2012; Cyster and Schwab, 2012), to be pharmacologically active (Figure 13). As a result, it mimics

the action of sphingosine-1-phosphate (S1P) by interacting with the class of G protein-coupled receptors named S1P receptors ($S1P_1$ and $S1P_{3-5}$, but not $S1P_2$), $S1P_1$ in particular, which is highly expressed on the plasma membrane of activated T lymphocytes (Figure 14).

In contrast with S1P, which modulates the diverse functions of a variety of cells through differential coupling of the receptor to heterotrimeric G-proteins (α_I, α_q or

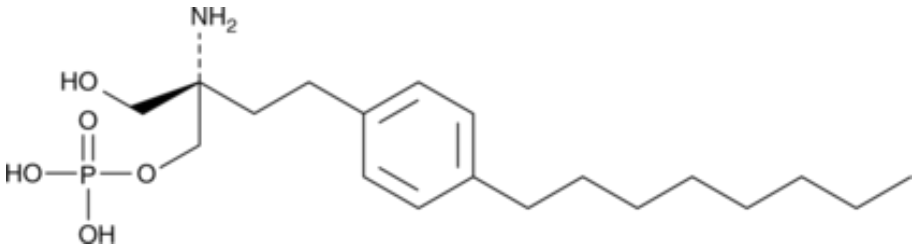

Figure 13. Phosphorylated form of fingolimod (FTY720)

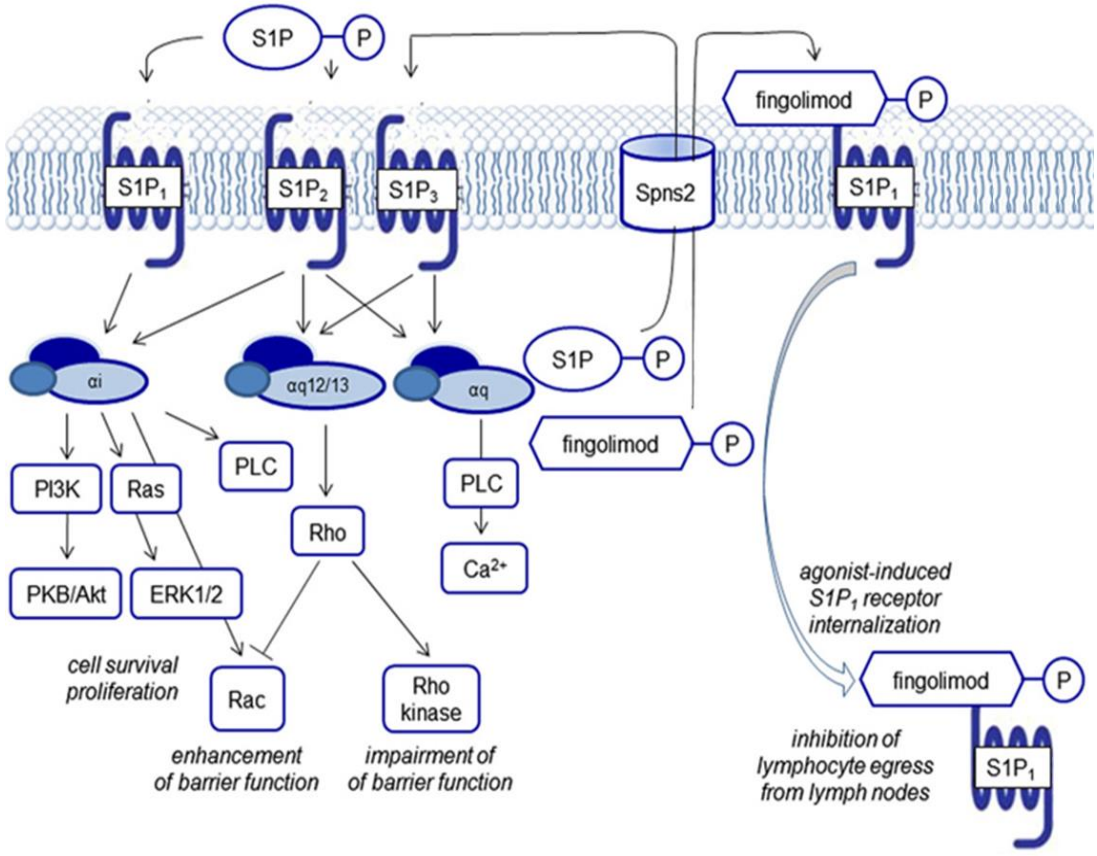

Figure 14. The phosphorylated form of fingolimod (FTY720) is extruded from the cell, thereafter binding to, and bringing about the internalization of S1P receptors, $S1P_1$ in particular (from Brinkmann et al., 2010).

$\alpha_{12/13}$) and through heterogeneity in terms of the constitutive and inducible patterns

of expression of the S1P receptors themselves in.immune cells (Rivera et al., 2008), FTY720 strongly binds to $S1P_1$ provoking its internalization and degradation rather than thereby preventing egression of T lymphocytes from lymphoid organs, which is the basis for the immunosuppression (Lee et al., 2010). Importantly, its ability to activate PP2A depends on its non-phosphorylated form, as demonstrated by the silencing of SK-2 (Saddoughi et al., 2013), which confers antitumor activity to FTY720, especially against hematologic malignancies (Oaks et al., 2013). The structure of FTY720 has recently been used as a template to design new molecules devoid of immunosuppressive action (Zonta et al., 2015), a characteristic highly desirable for the development of compounds that might be considered for pre-clinical studies or hopefully clinical trials including patients afflicted by any type of cancer. In our laboratories, new FTY720 analogues have recently been synthesized with a structure that retains specific features for the interaction with SET (Zonta et al., 2015). The preliminary successful tests have led to the development of a library of compounds that are now included in a patent application recently filed at the national patent office.

SRC HOMOLOGY 2 DOMAIN-CONTAINING PHOSPHATASE 1 (SHP-1)

Tyrosine phosphorylation, whether occurring upon engagement of Receptor Tyrosine Kinases (RTK) or being mediated as a result of the involvement of Non-Receptor Tyrosine Kinases (NRTK), is a critical event that initiates and regulates a myriad of cellular processes including growth factor receptor signaling, cell adhesion, metabolism, cell cycle control, transcriptional activation, to mention but a few (Hunter, 2009). This flow of information, which occurs through the integration of the great variety of signaling pathways being activated simultaneously, can be interrupted by the other actors of reversible tyrosine phosphorylation, namely protein tyrosine phosphatases (PTPs). As in the case of serine/threonine phosphatases, accumulating evidence shows that these are not merely the off-switches of signaling pathways, but contribute to the fine tuning of intracellular communication, so that the diverse stimuli yield generate specific effects that meet the cellular needs any time (Ostman and Böhmer, 2001). This multifaceted function can be well exemplified by the action of two members of the Src Homology 2 domain-containing Phosphatase (SHP) family 1 and 2, SHP-1 and SHP-2 for short (Neel et al., 2003; Chong and Maiese, 2007), which, though sharing high sequence homology, often play opposite biologic roles. As to their expression, SHP-1 is mainly present in hematopoietic and epithelial cells and is widely regarded as a negative regulator of signaling, whereas SHP-2 is ubiquitous and shown to have a role in promoting cell activation (Neef et al., 2006; Shaker et al., 2011; Thabut et al., 2011; Forbes et al., 2012). As to their role in disease, especially in cancer development, the absence of SHP-1 expression has been observed in several forms of lymphomas and leukemias (Ma et al., 2003; Neel, 1993; Wu et al.,2003), which underlines the role of SHP-1 as a tumor suppressor, whereas the uncontrolled activation of SHP-2 is considered an oncogenic factor (Irandoust et al., 2009).This chapter will focus on SHP-1, which is known as a negative regulator of signaling pathways downstream of several RTKs and now deemed a potential target for cancer therapy (Watson et al., 2016; López-Ruiz et

al., 2011). In CLL cells in particular, it has been recently demonstrated that SHP-1 is segregated into two forms, one in an active form being bound to the inhibitory co-receptor CD5, and the other in the cytosol in an inhibited conformation due to its phosphorylation of an inhibitory residue, both contributing to the resistance to apoptosis (Tibaldi et al., 2011).

SHP-1: structure

SHP-1 is encoded by the PTPN6 gene, the splicing of which gives rise to four 4 gene products, 3 variants of SHP-1 with different N-termini and a long form of SHP-1 termed SHP-1L where the C-terminus is extended. As shown in Figure 15A, the SH2 domains are situated at the N-terminus of the molecule, followed by the catalytic domain and a C-terminal tail. This latter is displayed in more detail in Figure 15B, where critical residues for the regulation of the catalytic activity are emphasized. In the inactive conformation, the N-terminal SH2 domain is

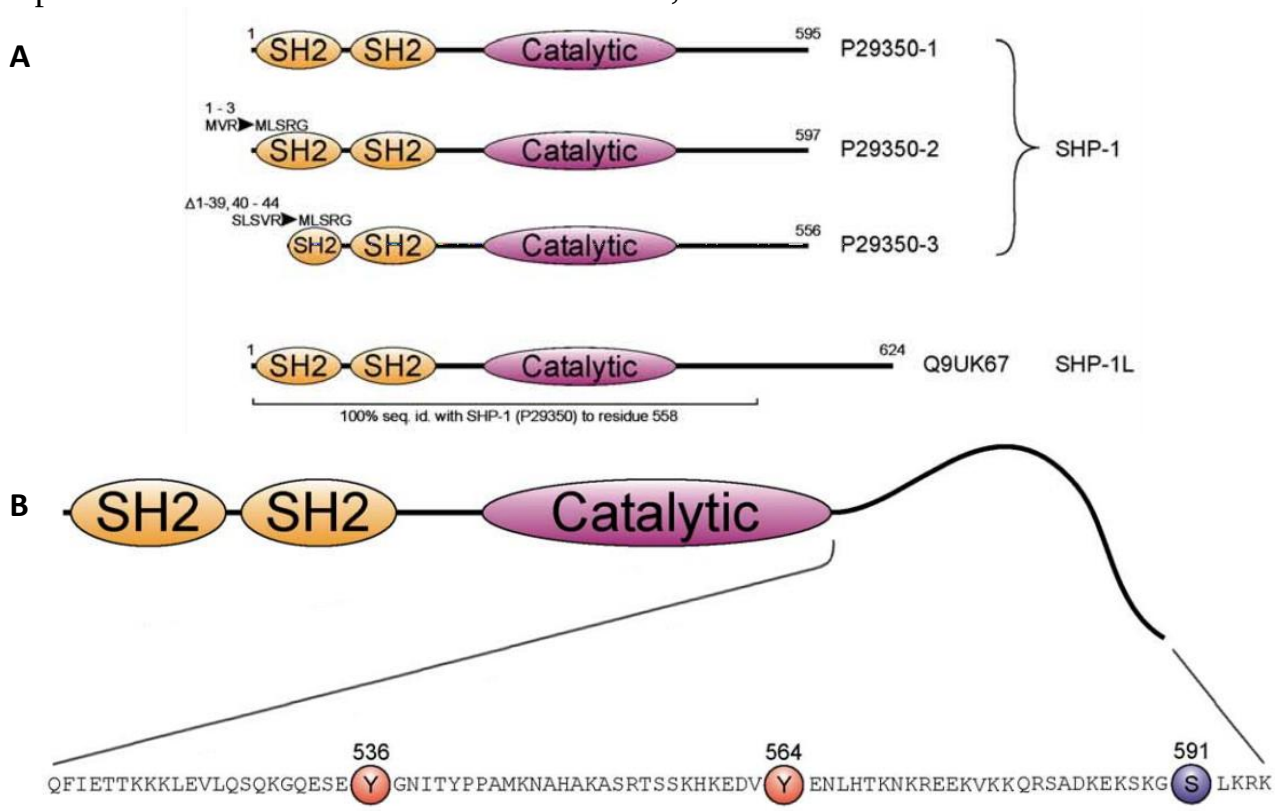

Figure 15. (A) Structural characteristics of SHP-1 variants (from Poole and Jones, 2005). **(B)** Structure of SHP-1 and sequence of the C-terminus with tyrosyl (Y, Tyr536 and Tyr564) and seryl (S, Ser591) residues playing a crucial role in the activation and inhibition of the phosphatase respectively.

intramolecularly associated with the PTP domain, thereby repressing its activity,

whereas the engagement of the SH2 domains leads to the activation of the phosphatase. Figure 16 shows that, whether due to the binding of the SH2 domains to the phosphorylated tyrosyl residues of Immunoreceptor Tyrosine Inhibitory Motifs (ITIMs, Figure 16A) in inhibitory cell surface co-receptors such as CD22 (Cyster and Goodnow, 1997), CD72 (Adachi et al., 1998), and CD5 (Tibaldi et al., 2011), or to the intramolecular interaction with the phosphorylated form of Tyr536 and Tyr 564 in the C-terminus (Figure 16B) (Zhang et al., 2003), the inhibition of SHP-1 is relieved. Besides the interaction of the N-terminal SH2 domain with the PTP domain, another mechanism of inhibition of SHP-1 is the phosphorylation of Ser591 by PKC or MAPK, which has not been described for SHP-2 (Jones et al., 2004; Poole and Jones, 2005).

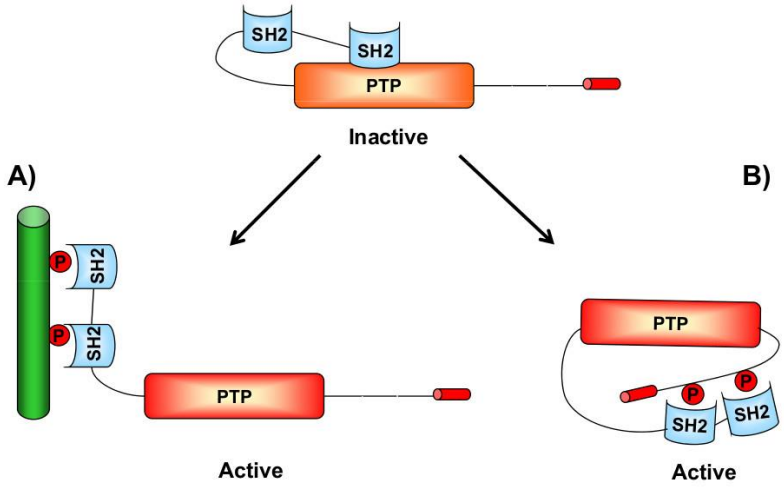

Figure 16. Models of the activation of SHP-1 based upon the engagement of the SH2 domains with phosphorylated tyrosyl residues of receptors or protein partners **(A)** or harbored within the C-terminus of the phosphatase. (from Poole and Jones, 2005).

SHP-1: function

Basically, SHP-1 is a regulator of haematopoietic cells, downregulating the pathways that promote cell growth and survival (Zhang et al., 2000), among others. In other types of cells, it also attenuates and terminates signals involved in cell proliferation, differentiation, survival, and apoptosis (Duchesne et al., 2003; Wu et al., 2003; Valencia et al., 1997; Keilhack et al., 2001; Tenev et al., 2000). SHP-1 also regulates glucose homeostasis by modulating insulin signaling in liver and

muscle (Dubois et al., 2006) and negatively affects bone resorption (Aoki et al., 1999).

SHP-1 performs these functions by either directly dephosphorylating the the cytosolic tails of the RTKs that become activated upon engagement with their respective ligands including Platelet-Derived Growth Factor Receptor (PDGFR), Insulin Receptor (IR), the Epidermal Growth Factor Receptor (EGFR), and Vascular Endothelial Growth Factor Receptor (VEGFR) type 2 (Geraldes et al., 2009; Dubois et al., 2006; Tenev et al., 1997; Bhattacharya et al., 2008), or components of the immune receptors that do not possess kinase activity such as from the activation of a variety of plasma membrane receptors including the B Cell Receptor (BCR) (Adachi et al., 2001), T Cell Receptor (TCR) (Perez-Villar et al., 1999), and NK Activating Receptor (Matalon et al., 2016). As previously mentioned and exemplified in Figure 17, this latter function occurs especially when SHP-1 is recruited to the ITIMs of the inhibitory receptors, such as CD5, CD22,

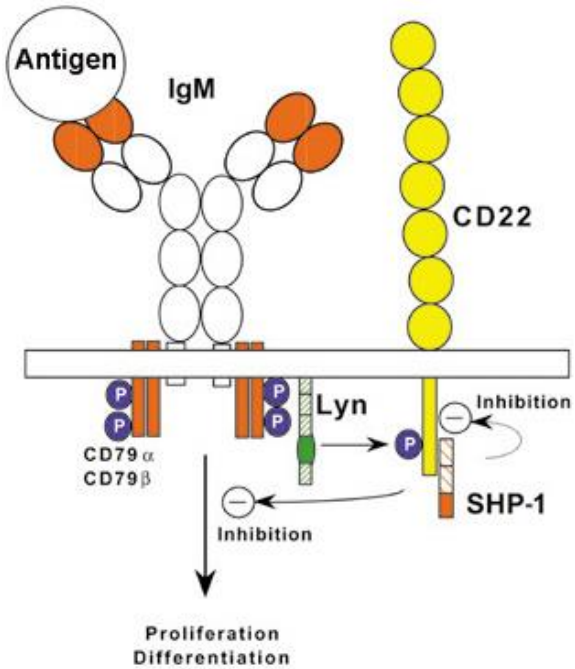

Figure 17. Role of SHP-1 in downregulating signals from plasma membrane receptors. The phosphorylation of the of inhibitory ITIMs of inhibitory receptors results in the recruitment of the cytosolic phosphatase SHP-1 with dephosphorylation of cytosolic tails of the plasma membrane receptors initiating signal transduction. Here, the action of SHP-1 on the signals downstream of BCR engagement is shown. Importantly Lyn is unique among the tyrosine kinases in immune cells, in that it takes part in negative feedback (from Silver and Cornall, 2003)

CD72, FcγRIIB, p70-NKB1 and KIR. (Zhang et al., 2000; Silver and Cornall,

2003), once these are uniquely phosphorylated by the SFK Lyn (Tibaldi et al., 2011). Moreover, SHP-1 downregulates signals mediated by non-receptor tyrosine kinases (especially those belonging to the Src family) and downstream mediators that participate in signal transduction such as Erk, JNK STATs, Jak2, NF-kB, and PI3K-Akt (Chong and Maiese, 2007).

The current understanding of the highly complex function of SHP-1 has posed the question whether it might represent a new attracting target for drug development, especially in diseases where its activity is suppressed, as in the case of several types of cancer (Sharma et al., 2016; Watson et al., 2016). Importantly, drugs already used in the clinical practice as multi-kinase inhibitors have recently proved to be direct activators of SHP-1, the molecular models showing that these compounds may intervene in the inhibitory mechanism mediated by the N-terminal SH2 domain (Tai et al., 2014a). In the following sections, more details will be provided as to their interaction with SHP-1 and their potential role as templates for the development of more selective compounds to be employed in cancer therapy.

SHP-1 activators: angiokinase inhibitors doing a front flip?

In the past few years, drugs already approved as multi-angiokinase inhibitors directed against RTKs such as PDGFR and VEGFR in particular, have emerged as agents capable of activating SHP-1, including sorafenib (Figure 18A) (Wilhelm et al., 2008) and nintedanib, also known as BIBF-1120 (Figure 18B) (Tai et al., 2014a). Many derivatives of the former have been developed and tested in a number of cancer models, including hepatocellular carcinoma (Tai et al, 2014b) and triple negative breast cancer cells (Liu et al., 20117), exploiting this phosphatase-directed potential. As to nintedanib, it has been approved for the treatment of pulmonary fibrosis and second-line therapy of lung adenocarcinoma (McCormack, 2015) and now being evaluated in further clinical trials. Interestingly, a docking model has been proposed for nintedanib targeting SHP-1, based upon the interaction with Glu524 through hydrogen bonding (Tai et al., 2014a), residue that lies in the close proximity to the catalytic domain of the phosphatase and may account for a steric

hindrance preventing the inhibited conformation promoted by the N-terminal SH2 domain. In our leukemia model, we also had observed that the phospho-Ser591-

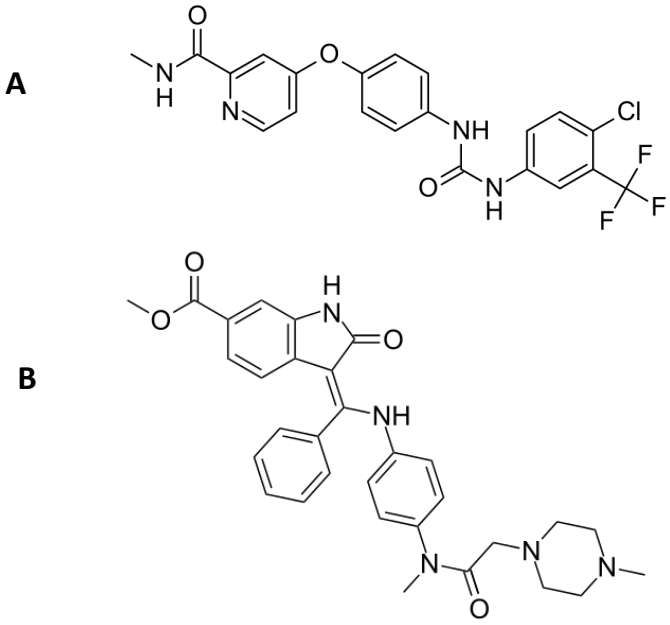

Figura 18. Sorafenib (A) and nintedanib (B)

dependent inhibition occurred, which , according to other authors (Tai et al., 2014a) can be circumvented by nintedanib itself, which operates independently of post-translational regulatory mechanisms, possibly driving conformational changes with subsequent activation of the phosphatase. In Figure 19, the docking model describing the SHP-1/nintedanib interaction is shown.

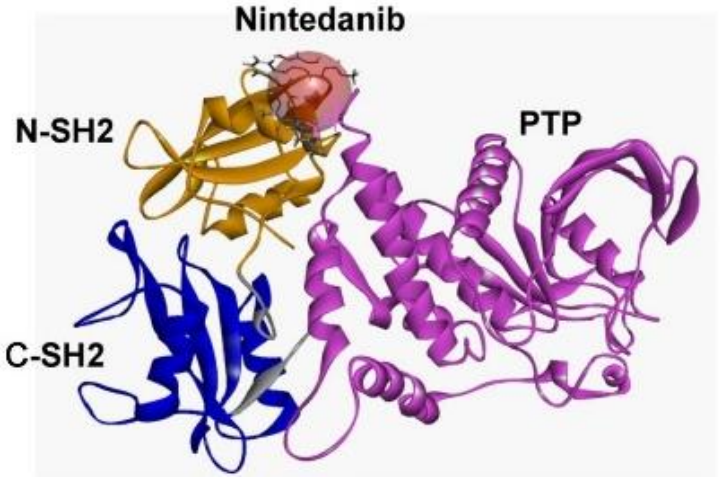

Figura 19. Model of interaction between nintedanib and SHP-1

Notably, these studies show that a crucial target for SHP-1 is the phosphorylated form of the family of Signal Transducer and Activator of Transcription (STAT) proteins, and STAT3 in particular, whose activity as cytoplasmic signaling protein and nuclear transcription factor is frequently elevated in a variety of solid tumors and hematological malignancies and which is abolished by the treatment with sorafenib, nintedanib or derivatives thereof (Yang et al., 2008; Abdulghani et ., 2013; Su et al., 2016; Chen et al., 2012; Tai et al., 2014a).

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RATIONALE OF THE STUDY

This study draws upon previous observations concerning the functional status of critical enzymes in the signal transduction of CLL cells, which directly depends on the phosphorylation of specific factors at regulatory residues and in specific compartments as well as the interaction with protein partners that affect their activity. More in detail, having assessed that the inhibitory seryl residue of SHP-1 is dephosphorylated by PP2A (Zonta et al., 2015), the aim of this work was

- to assess whether he restoration of PP2A activity by the FTY720 analogue MP07-66 might lead to SHP-1 activation, resulting in the suppression of the Lyn-dependent pervasive tyrosine phosphorylation that characterizes CLL cells and is shown to be an anti-apoptotic factor;
- to attempt to activate SHP-1 directly by using nintedanib, which in addition to acting as a triple angiokinase inhibitor, is capable of circumventing the phospho-S591-dependent inhibition of the phosphatase;
- to establish whether the activation of SHP-1 affected the level of phosphorylation of $PP2A_C$ at Tyr307, a residue that was shown to be crucial for a tight interaction of PP2A with SET and the total inhibition of the phosphatase activity of PP2A itself, and this effect might ignite a virtuous cycle that could potentiate the action of both of these tumor suppressors.

In addition to providing further insight into the molecular basis of CLL, the results achieved in this study might put forward new prospects in the development of alternative strategies in the treatment of CLL by mobilizing endogenous resources (phosphatases), which are typically inhibited in cancer cells rather than suppressing active players (*e. g.* kinases), which may be operating in normal tissues at the same time, with consequent adverse undesidered effects.

EXPERIMENTAL SECTION

Nintedanib directly activates SHP-1 in the cytosol of CLL cells

We previously demonstrated that SHP-1 is present in CLL cells in two forms, one bound to the plasma membrane receptor CD5 in an active state, and the other in the cytosol in an inhibited conformation (Tibaldi et al., 2011). As shown in Figure 20A, the plasma membrane-enriched fraction (particulate) and the cytosol of CLL cells were separated from total lysates and immunoblotted with anti-pY536-SHP-1 and anti-pS591-SHP-1 antibodies, a positive response to which is indicative of either activation or inhibition of SHP-1, respectively. As expected, the distribution of SHP-1 in the two cellular compartments paralleled the functional status, these characteristics turning out to be independent of the diverse biological and clinical features of the single patients (Table 2). Moreover, in order to establish how the phosphorylation status affected SHP-1 activity, SHP-1 was pulled down from the cytosolic and particulate fractions and tested for the phosphatase activity by using ^{32}P-labeled Band 3 as a substrate (Tibaldi et al., 2014). The phosphatase activity of SHP-1 exhibited by the cytosolic fraction was negligible as compared to that of the particulate (Figure 20B), underscoring that the catalytic activity of SHP-1 can be profoundly affected by the phosphorylation at different residues. This finding raised the question whether the inhibition of the pool of SHP-1 in the cytosol was related to the elevated level of tyrosine phosphorylation CLL cells in this compartment, which was previously shown to characterize this disease and to promote anti-apoptotic mechanisms (Zonta et al., 2015). Therefore, we first performed in-vitro phosphatase activity assays on the cytosolic pool of SHP-1 in the presence of nintedanib (Figure 20), which in addition to acting as receptor tyrosine kinase inhibitor (Hilberg et al., 2008), (Roth et al., 2009) has been shown

to remove the pS591-dependent inhibition of SHP-1 (Tai et al., 2014). To this end,

Table 2 . Biological and clinical characteristics of the patients

Patient no.	Age, y	Sex	Rai stage*	WBC count/mm^3	Lymphocytes, %	V_H mutational status†	Zap-70 expression‡	FISH
1	84	F	0	48630	95,2	Unmut	POS	13q-
2	78	M	0	34910	62,7	Unmut	POS	11q-;13q-;12+
3	65	M	0	79430	98,5	Mut	POS	13q-
4	57	F	0	109500	97	Unmut	NEG	13q-
5	56	M	0	85150	89	Unmut	NEG	11q-;12+
6	81	M	0	112000	80,8	Mut	POS	13q-
7	89	F	0	50300	63	Unmut	POS	nd
8	65	M	0	51180	96,8	Mut	POS	13q-
9	79	F	I	53400	76	Mut	NEG	13q-
10	69	F	I	45150	94,4	Mut	NEG	13q-
11	54	M	I	57110	82,6	Unmut	NEG	12+
12	67	F	I	61660	92,9	Unmut	NEG	12+
13	76	M	I	158000	97	Mut	NEG	N
14	82	M	I	31010	51,2	Mut	POS	12+
15	82	F	I	145700	95	Unmut	POS	12+
16	67	F	I	61660	92,9	Unmut	NEG	12+
17	77	M	I	35140	83,5	Unmut	NEG	17p-;11q-
18	64	F	I	57600	93	Mut	NEG	13q-
19	73	F	I	58100	83,9	Mut	NEG	13q-
20	73	F	II	35990	82	Mut	POS	nd
21	66	F	II	47180	92,6	Unmut	POS	13q-
22	76	M	II	55600	87	Unmut	POS	17p-
23	76	M	II	25290	76	Mut	NEG	N
24	64	M	II	74610	89	Mut	POS	13q-
25	66	M	II	99600	91	Unmut	NEG	11q-
26	77	F	II	62110	84	Unmut	NEG	13q-
27	80	F	II	155800	94,5	Mut	NEG	N
28	63	M	III	93290	91,3	Mut	NEG	12+
29	77	F	III	43290	93,9	Mut	POS	13q-
30	63	M	III	90170	85,5	Mut	NEG	12+
31	76	M	IV	122000	94	Unmut	NEG	13q-
32	79	M	IV	69370	83	Unmut	NEG	N
33	72	M	IV	91950	95	Mut	POS	13q-
34	69	M	IV	65600	89	Mut	NEG	17p-;13q-
35	64	M	IV	97970	63	Mut	POS	13q-;12+
36	83	F	IV	26590	91	Unmut	POS	13q-
37	57	F	IV	82520	55	Unmut	NEG	N

NEG, negative; POS, positive; WBC, white blood cell; N, CLL with a normal karyotype; nd, not determined.

* Staging system developed by Rai et al.

†Mutated was defined as having a frequency of mutations >2% from germline V_H sequence.

‡ As determined by western blot analysis on purified B cells (purity at least 98%).

SHP-1 was pulled down from the cytosolic fraction of CLL cells in the absence or the presence of serine/threonine phosphatase inhibitors in order to assess the effect of increasing concentrations nintedanib on the activity of the phosphorylated and

non-phosphorylated forms of SHP-1 (Figure 20C, inset). Figure 20C shows that

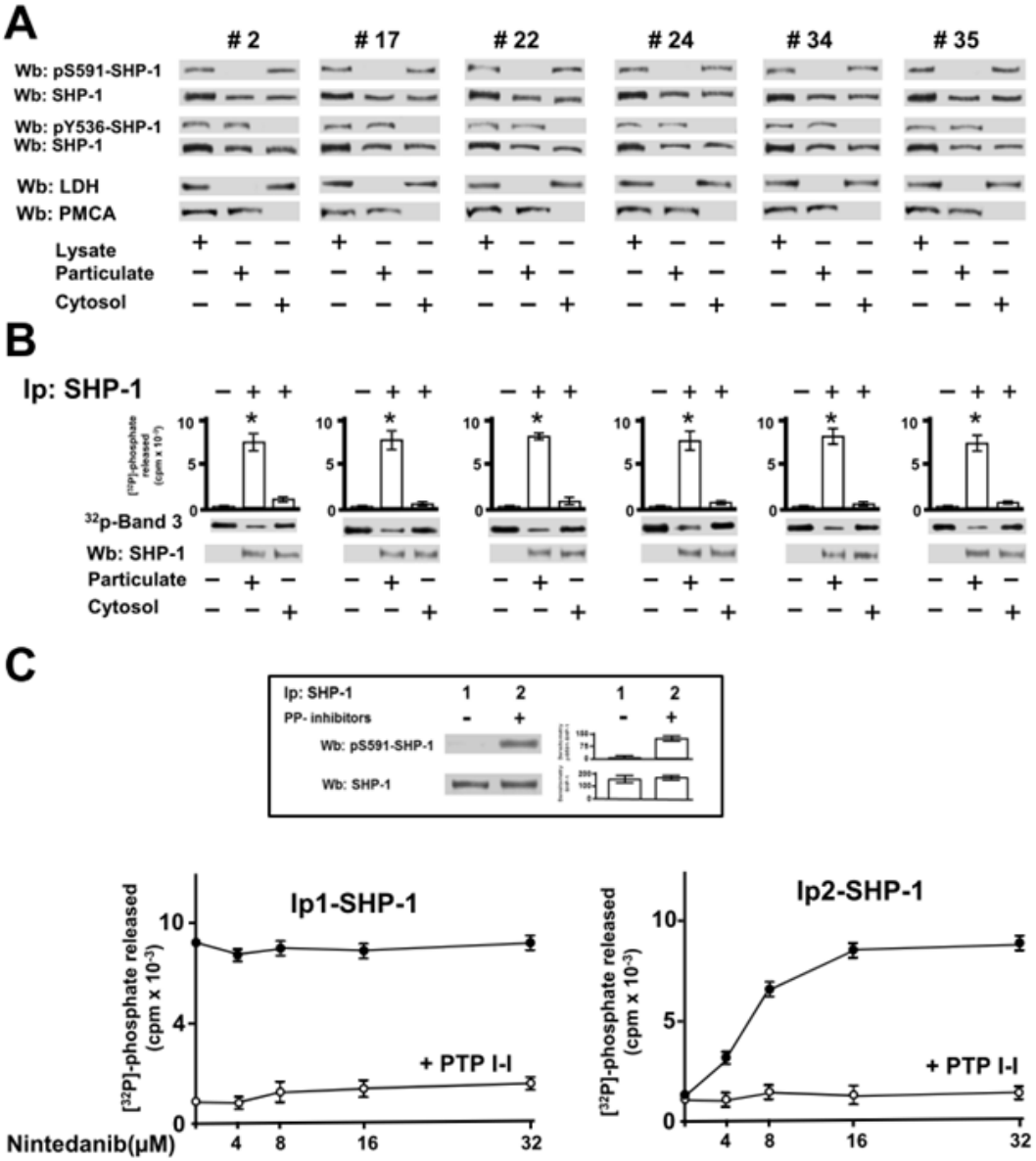

Figure 20. In-vitro effect of nintedanib on the differently phosphorylated forms of SHP-1 pulled down from CLL cells. **(A)** Whole cell lysates, particulate and cytosol of 5 x 10^5 CLL cells underwent Western blot (Wb) analysis with anti-pS591-SHP-1 and pY536-SHP-1 antibodies and reprobed with anti-SHP-1, anti-LDH (cytosolic marker) and anti-PMCA (plasma membrane marker) antibodies as loading controls. #2, #17, #22, #24, #34 and #35 are patients belonging to various clinical and biological subtypes. **(B)** Tyrosine phosphatase activity of SHP-1 immunoprecipitates recovered from particulate and cytosol of CLL cells. **(C)** Tyrosine phosphatase activity of SHP-1 immunoprecipitated from the cytosol of CLL cells of 15 patients in the absence (Ip1-SHP-1) and presence (Ip2-SHP-1 1) of serine/threonine phosphatase inhibitors and determined in vitro in the presence of increasing concentrations of nintedanib supplemented without (solid circles) or with 25 μM PTP I-I (open circles). The inset shows the level of pS591-SHP-1 determined by Wb analysis and reprobed, after stripping, with anti-SHP-1 antibody as loading control pertaining to from the patient #34 (left-hand panel) and the pooled densitometric analysis (arbitrary units, right-hand panel) of the patients examined. The data are the mean ± SD of 3 experiments performed in triplicate (*P ≤ 0.01).

nintedanib was capable of activating the phosphorylated, and inhibited, form of

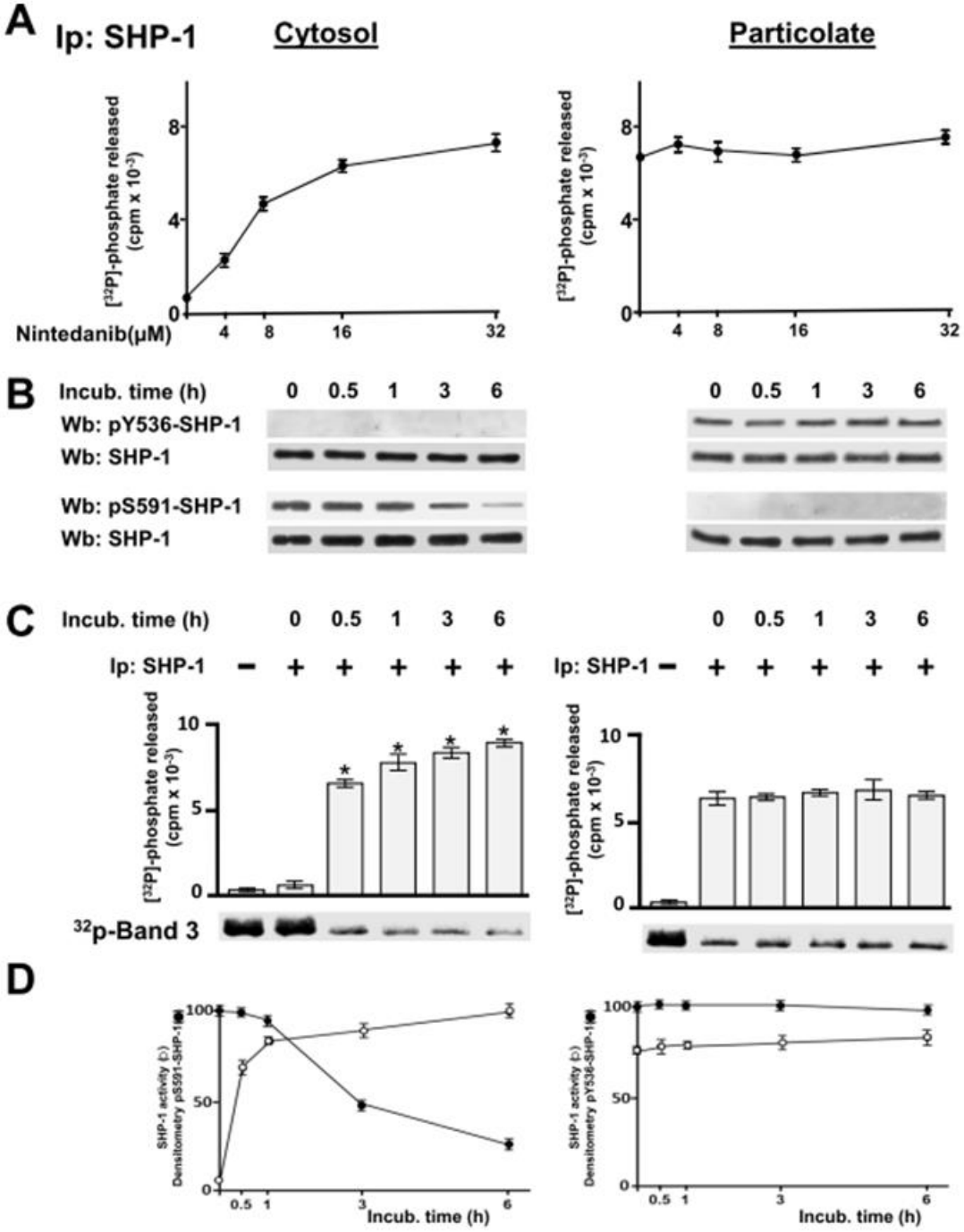

Figure 21. Effect of nintedanib on the activity of the differently phosphorylated forms of SHP-1 inside CLL cells (A) The activity of SHP-1 immunoprecipitated from cytosol (left-hand panel) and particulate (right-hand panel) of freshly isolated CLL cells cultured in the absence or presence of increasing concentrations of nintedanib for 1 h was measured as [^{32}P]phosphate released from [^{32}P]phospho-band 3. **(B)** Wb analysis of cytosol and particulate from freshly isolated CLL cells were cultured in the presence of 15 μM nintedanib over time performed with pS591-SHP-1 and pY536-SHP-1 antibodies. **(C)** SHP-1 immunoprecipitated from the cytosol (left-hand panel) or particulate (right-hand panel) of the CLL cells described in (B), was tested for the basal tyrosine phosphatase activity in vitro on [^{32}P]phospho-band 3**. (D)** Densitometric analysis of Western blots probed with anti-pS591 or alternatively anti-pY536 antibody of all the 16 patients (arbitrary units, open circles, left- and right-hand panel respectively) as well as the values of the phosphatase activity reported as histograms in (C), normalized as percentage (solid circles, left- and right-hand panel respectively). The data are the mean ± SD of 3 experiments performed in triplicate (*P ≤ 0.01).

SHP-1 (Ip2-SHP-1, right-hand panel), as expected on the basis of previous data

(Tai et al., 2014), whereas the non-phosphorylated form was not influenced by the compound in that the phosphatase was already active (Ip1-SHP-1, left-hand panel). These results appear to be in line with the hypothesis that this drug causes a change in the inhibited conformation of SHP-1 induced by the phosphorylation at S591. Similarly, we analyzed the activity of SHP-1 immunoprecipitated from the cytosolic and particulate fractions of CLL cells treated with increasing concentrations of nintedanib, considering that SHP-1 in these compartments is differently phosphorylated and active. As shown in Figure 21A, SHP-1 reached a full activation at a concentration as high as 15 μM nintedanib (left panel), as determined by measuring the dephosphorylation of ^{32}P-band 3, whereas the one from the particulate was unaffected (right panel). After incubating CLL cells over time at 15 μM nintedanib, we evaluated the phosphorylation status of SHP-1 on the cytosolic and particulate fractions by Western blot analysis with anti-pS591-SHP-1 and anti-pY536-SHP-1 antibodies, respectively, and performed phosphatase activity assays on SHP-1 after immunoprecipitation at each time interval. Nintedanib affected neither the phosphorylation status (Figure 21B, right-hand panel), nor the catalytic activity (Figure 21C, right-hand panel) of the SHP -1 pool of the particulate, whereas the one in the cytosol was readily activated by nintedanib, reaching the maximal efficacy already at the earliest incubation times by circumventing the inhibitory phosphorylation at S591 (Figures 21B and 21C, left-hand panel). The data obtained by Western blot analysis and phosphor imaging were quantitated as arbitrary units and sketched in Figure 2D for clarity's sake.

Nintedanib induces activation of caspase-8 and PP2A by decreasing tyrosine phosphorylation in CLL cells

We previously showed that the cytosol of CLL cells is characterized by the elevated level of tyrosine phosphorylation promoted by the aberrant constitutive activity of HSP90-bound Lyn, promoting anti-apoptotic mechanisms (Trentin et al., 2008). To verify whether the cytosolic tyrosine phosphorylation in CLL cells was related to

the inhibition of SHP-1 in this compartment, SHP-1 was silenced in freshly isolated leukemic B cells, which were subsequently incubated for 1 hour in the presence of increasing concentrations of nintedanib (0-30 μM), lysed and analyzed by Western blot analysis with anti-phosphotyrosine antibody. Nintedanib caused a dramatic reduction in tyrosine phosphorylation at a concentration of 10 μM (Figure 22A and

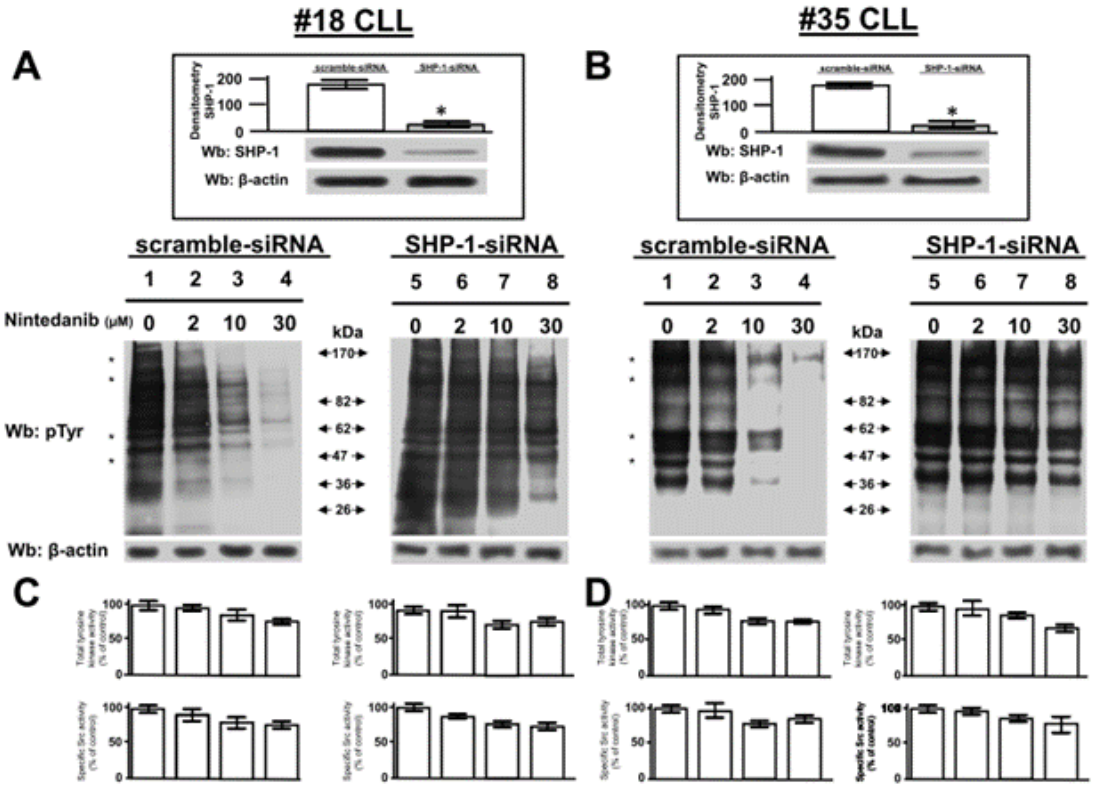

Figure 22. Effect of nintedanib on the tyrosine phosphorylation of CLL cells. Freshly isolated CLL cells were transfected with either scramble RNA or SHP-1-siRNAs and incubated for 48 h in complete medium and the expression of SHP-1 was determined by Wb analysis (pooled densitometric analysis of all the samples tested are histogrammed in the top panel of the insets). The immunoblots of two samples from patients #18 **(A)** and #35 **(B)** are shown in the bottom panel in the insets. CLL cells were subsequently cultured in the absence or presence of increasing concentrations of nintedanib for 1 hour and whole cell lysates were analyzed by Wb analysis with anti-pY antibody. The Wb strips show the results concerning the patients #18 and #35 and are representative of all the patients examined. **(C, D)** The global tyrosine kinase activity and the specific Src activity in the CLL cells cultured as in (A) and (B) was determined by using the nonspecific random polymer polyGlu$_4$Tyr (top panel) or the specific peptide substrate cdc2(6-20) (bottom panel) and the pooled data are reported as histograms. The data are expressed as mean ± SD of 3 experiments performed in triplicate (*P ≤ 0.05).

22B, left panels), which was significantly high er than the nanomolar range reported to inhibit the receptor tyrosine kinases (RTKs) that nintedanib is known to target (Hilberg et al., 2008;), (Roth et al., 2009). Importantly, its effect was

abrogated by the genetic inhibition of the phosphatase itself (Figure 22A and 22B, right panels), and proved independent, if not to a very limited extent, to the inhibition of tyrosine kinases as assessed by tyrosine kinase activity assays using poly-Glu-Tyr or cdc2[6-20], peptide substrates used to determine the global (including that of RTKs) or the SFK specific activity, respectively (Gringeri et al., 2009), (Tibaldi et al., 2011a). In fact, as histogrammed in Figure 22C and 22D, both types of activities were affected at most by 30% of the control only at high concentrations of nintedanib (over 10 μM). The hypothesis that the effect observed was mediated by the activation of SHP-1, rather than the inhibition of a tyrosine kinase, was further supported by in vitro kinase assays showing an IC50 of 12 μM nintedanib for Lyn at 20 μM ATP, whereas dasatinib, a SFK inhibitor, exhibited an IC50 of 50 nM (data not shown). Additionally, we wanted to evaluate whether nintedanib could impinge upon CLL cell viability, considering that the apoptosis of CLL cells was already found to be related to the drug-induced decrease in tyrosine phosphorylation (Contri al., 2005; Trentin et al., 2008). Therefore, fresh B cells isolated from CLL patients were incubated with increasing concentrations of nintedanib for 24 and 48 h and then subject to annexin V-PI flow cytometry. As shown in Figure 23A, the level of apoptosis reached 50% and 60%, at 24 hours and 48 hours, respectively, at a nintedanib concentration as high as 10 μM (left-hand panel), with concomitant cleavage of PARP and caspase-3, markers of caspase-dependent apoptosis (right-hand panel). Similar results were obtained by co-culturing CLL cells with bone marrow mesenchymal stromal cells (data not shown) so as to mimic the tissue microenviroment where CLL cells proliferate (Ding et al., 2010).

To further investigate the contribution of SHP-1 to the nintedanib-induced apoptosis, we used genetic and pharmacological inhibition of the phosphatase itself. Figure 23B (left-hand panel) shows that the number of apoptotic cells after 24 hours' treatment with nintedanib (bar 4) was dramatically reduced by the silencing of SHP-1 (bar 8) to levels comparable to those reached by knocking down the phosphatase in the absence of nintedanib (bar 6). At the molecular level,

nintedanib induced casp ase-dependent apoptosis, as witnessed by the cleavage of

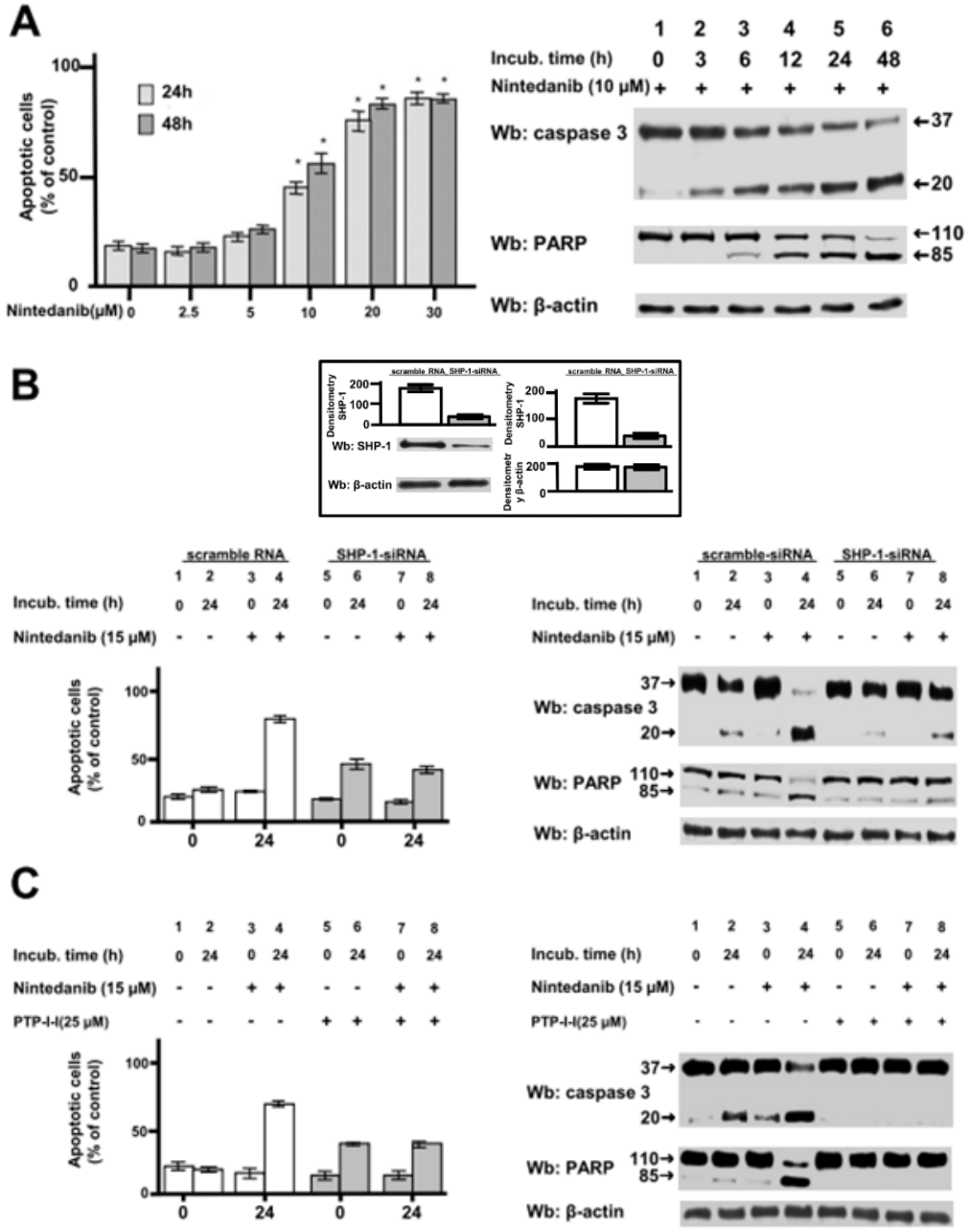

Figure 23. Effect of nintedanib on the viability of CLL cells. CLL cells underwent annexin V–PI flow cytometry after all the treatments and quadrant analysis after flow cytometry was histogrammed and expressed as mean percentage of early and late apoptosis ± SD from three separate experiments performed in triplicate. 5 x 10^5 CLL cells fro each experiment were lysed and analyzed by Wb analysis with anti-caspase 3 and anti-PARP antibodies to monitor caspase-dependent apoptosis, and anti-β-actin antibody as a loading control (right panels). **(A)** Apoptosis of freshly isolated CLL cells cultured in the absence or presence of increasing concentrations of nintedanib for 24 and 48h. Compared with the control, changes were statistically significant (left panel, *P ≤ 0.01). **(B)** After transfection of CLL cells with either scramble RNA (white bars) or SHP-1-siRNAs (grey bars) for 48 h in complete medium, the expression of SHP-1 was determined by Wb analysis The data are the mean ± SD of 3 experiments performed in duplicate. *P ≤ 0.01. CLL cells were subsequently incubated for 0 or 24 h in the absence (bars 1-4) or presence (bars 5-8) of 15 μM nintedanib. Compared with the control, changes were statistically significant (left panel, *P ≤ 0.01). **(C)** Freshly isolated CLL cells were incubated for 0 or 24 h in complete medium in the absence (bars 1-4) or presence (bars 5-8) of 25 μM PTP-I-I and supplemented without (bars 1-2 and 5-6) or with (3-4 and 7-8) 15 μM nintedanib.

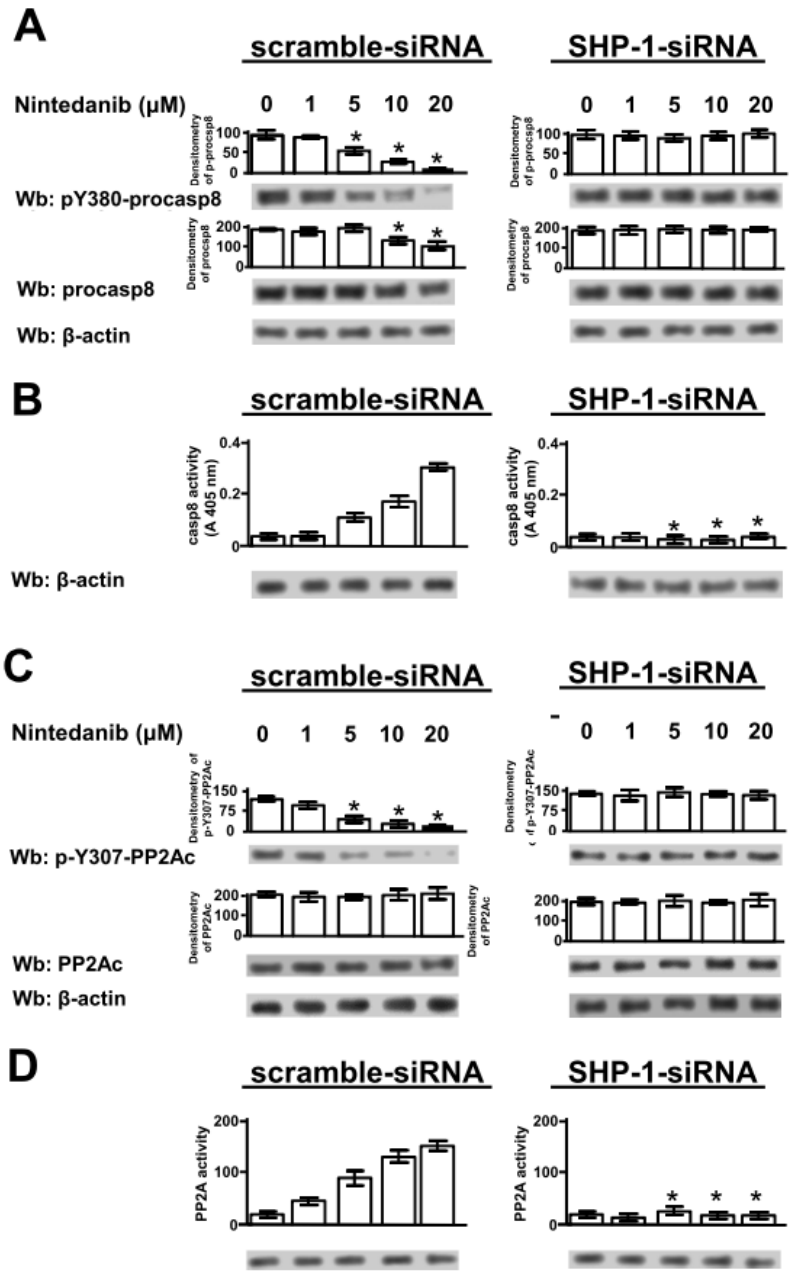

Figure 24: Effect of nintedanib on the phosphorylation state and activity of procaspase 8 and PP2Ac. Freshly isolated CLL cells were transfected with either scramble RNA (left-hand panels) or SHP-1-siRNAs (right-hand panels), cultured for 48 h in complete medium and subsequently cultured in the absence or presence of increasing concentrations of nintedanib for 1h. **(A)** After the 6 hours' treatment described above, cells were lysed and underwent Wb analysis with anti-pY380-procasp8 antibody. The blots were then stripped and reprobed with anti-procasp8 antibody and with anti-β-actin antibody as a loading control. Densitometric analysis (arbitrary units) of the pY380-procasp8 and pro-casp8 bands are reported as histograms. Data are expressed as mean ± SD from 4 experiments performed in triplicate. **(B)** After the 6 hours' treatment described above, cell lysates were assayed for *in vitro* casp8. Compared with the effect of nintedanib, changes due to PTP I-I were statistically significant ($*P \leq 0.01$). **(C)** After the 1 hours' treatment described above, cells were lysed and analyzed by Wb analysis with anti-pY307-PP2Ac antibody. The blots were then stripped and reprobed with anti-PP2Ac antibody and with anti-β-actin antibodies as a loading control. Densitometric analysis (arbitrary units) of the pY307-PP2Ac and anti-PP2Ac bands are histogrammed. Data are expressed as mean ± SD from 4 experiments performed in triplicate. **(D)** After the 6 hours' treatment described above, cell lysates wereassayed for *in vitro* PP2A activity by using a specific phosphopeptide as a substrate. Compared with the effect of nintedanib, changes due to PTP I-I were statistically significant ($*P \leq 0.01$). The data in the figure show the results concerning the patient #31 and are representative of all the patients examined.

caspase-3 and PARP (Figure 23B, right-hand panel, lane 4), the latter event being negligible when silencing SHP-1, irrespective of the presence of nintedanib (lanes 6 and 8). These findings were consistent with our previous data demonstrating that SHP-1 knock-down brings about caspase-independent apoptosis by targeting the plasma membrane pool of the phosphatase, which is catalytically active and orchestrates anti-apoptotic signals (Tsui et al., 2006; Tibaldi et al., 2011). The

pharmacological inhibition of SHP-1, achieved by the use of PTP-I-I, provided results overlapping those obtained by using SHP-1 siRNAs (Figure 22C). These observations led us to hypothesize that the dephosphorylation of specific SHP-1 substrates underlay the induction of apoptosis following treatment with nintedanib in CLL cells. Therefore, we focused our attention on two factors that we had previously explored and the activity of which we found to be inhibited in CLL via phosphorylation by the aberrant cytosolic form of Lyn, namely the caspase-8 zymogen (procaspase-8) (Zonta et al., 2014) and the serine/threonine Protein Phosphatase 2A (PP2A) (Zonta et al., 2015). After incubating freshly isolated CLL cells with increasing concentrations of nintedanib, we performed Western blot analysis with antibodies directed against the phosphorylated form of specific inhibitory residues of these two proteins, Y380 of procaspase-8 and Y307 of the catalytic subunit of PP2A (PP2Ac), respectively. Both tyrosines were phosphorylated when SHP-1 was not silenced nintedanib was not added, (Figure 24A and 24C, left-hand panels, 0 μM), the level of phosphorylation gradually declining as nintedanib concentration increased (1-20 μM). Moreover, total lysates from the same samples were assayed for the activity of the two enzymes in vitro by using commercial kits (see Materials and Methods for details), which allowed us to conclude that the dephosphorylation induced by nintedanib paralleled the level of activation of both enzymes (Figure 24B and 24D, left-hand panels). Importantly, dephosphorylation and activation were blocked, albeit in the presence of nintedanib, by subjecting CLL cells to SHP-1 knock-down, (Figure 24, right-hand panels), indicating that procaspase-8 and PP2Ac are substrates for SHP-1 and effectors of a SHP-1- dependent pro-apoptotic pathway.

Apoptosis of the CLL cells can be induced by indirect activators of SHP-1

The data collected thus far confirmed that nintedanib could trigger SHP-1 by circumventing the phosphorylation at the inhibitory residue S591. Interestingly, we recently demonstrated that pS591 could be dephosphorylated by PP2A, the activity of which was impaired by the phosphorylation at Y307 as well as the interaction

with its endogenous inhibitor SET and restored by a fingolimod analogue devoid of immunosuppressive action, the so-called MP07-66 (*2,2-diethoxyethyl{[4-(hexyloxy)phenyl]methyl})amine*) (Zonta et al., 2015). This latter event was shown to underlie the activation of apoptotic cascades based on the dephosphorylation of PP2A substrates (Zonta et al., 2015). These findings led us to conjecture that MP07-66 could be used as an indirect activator of SHP-1 via PP2A, and exploited to potentiate the action of nintedanib on SHP-1 itself in order to reinforce the apoptotic response of CLL cells. As shown in Figure 25A (left-hand panel), incubation with increasing concentrations of MP07-66 (0-15 μM) brought about apoptosis in 50% and 80% of CLL cells, at 24 hours and 48 hours, respectively, as determined by annexin V–PI flow cytometry. Of note, a time-course analysis of the cleavage of PARP indicated that apoptosis was dependent on caspase activation (right-hand panel). To evaluate whether SHP-1 was implicated in these events, aliquots from the cytosol for each time interval were immunoblotted with anti-pS591 SHP-1 antibody and tested for phosphatase activity in the presence of ^{32}P-labeled Band 3, showing that the dephosphorylation of SHP-1 was concomitant with the elevation of the activity thereof as the concentration of MP07-66 increased (Figure 25B and 25C). Further evidence for the role of SHP-1 in mediating apoptosis of CLL cells upon treatment with MP07-66 was provided by Western blot analysis with antibodies against pY380-procaspase8 and pY307-PP2Ac, revealing the dephosphorylation of the inhibitory residues (Figure 25D, lane 6) at 6 h. As expected, this event was blocked by the specific SHP-1 inhibitor PTP-I-I (Figure 25D, lane 8) as well as by okadaic acid (lane 7), which confirmed that the activation of PP2A drives the dephosphorylation of SHP-1 S591, in turn promoting global tyrosine dephosphorylation and cell demise. This latter observation is consistent with the hypothesis that SHP-1 and PP2A form a signalling axis wherein the single phosphatases, when stimulated, can activate one another, and even more so that such process can be amplified by using a combination of molecules activating both at the same time.

MP07-66 potentiates the pro-apoptotic effect of nintedanib

Since our data suggest that the activation of either PP2A or SHP-1 triggered by specific small molecules results in stimulating each other's activity and igniting a positive feed-back signalling loop that promotes apoptosis, we wondered whether the combination of nintedanib and MP07-66 could result in a more robust apoptotic response of CLL cells. Therefore, freshly isolated CLL were incubated with 15 µM nintedanib and 8 µM MP07-66 at different times and apoptosis was monitored

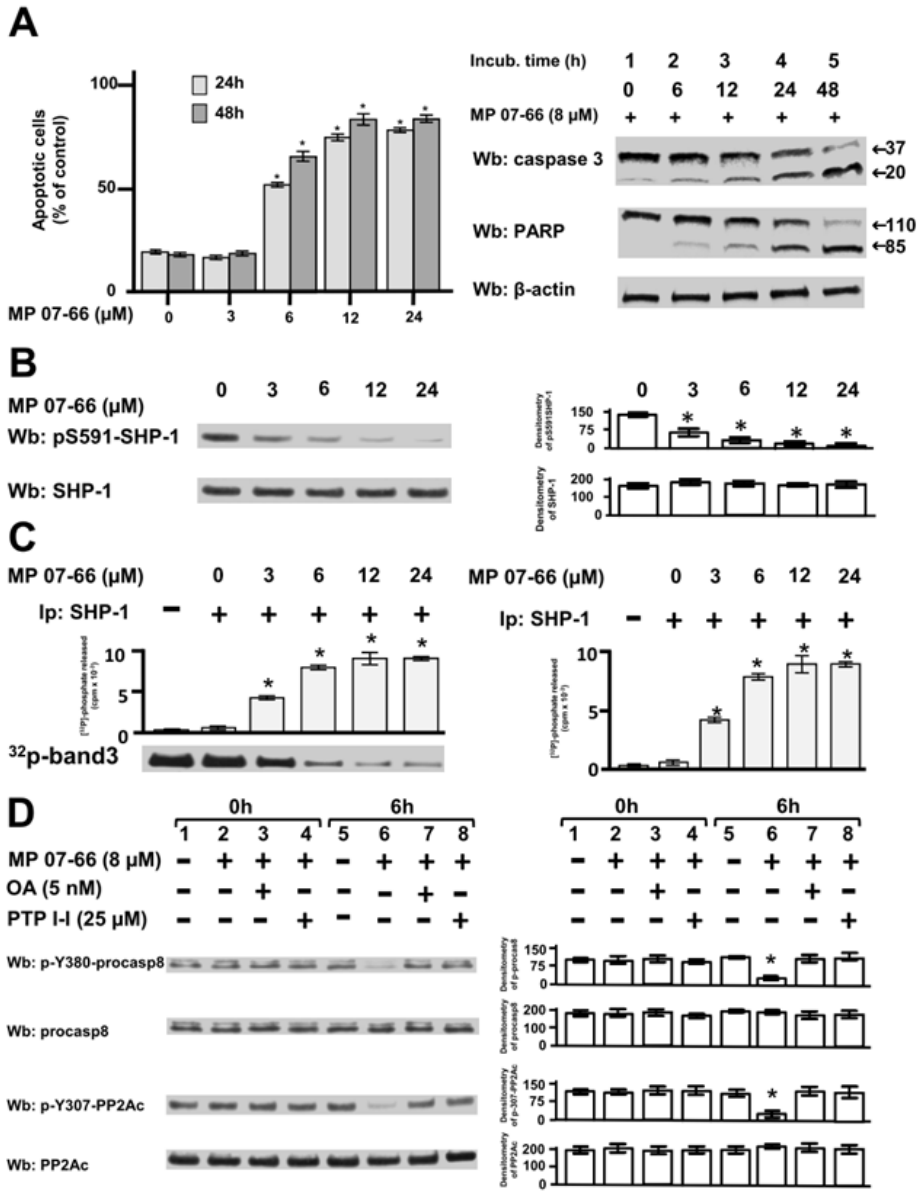

Figure 25: Effect of MP 07-66 on the CLL cell survival. (A) Freshly isolated CLL cells were cultured in the absence or presence of increasing concentrations of MP07-66 for 24 h and 48 h. After such treatment, cell apoptosis was analyzed by annexin V–PI flow cytometry. Quadrant analysis after flow cytometry was histogrammed and expressed as mean percentage of early and late apoptosis ± SD from three separate experiments performed in triplicate. Compared with the control, changes due to MP07-66 were statistically significant (*P ≤ 0.01). 5 x 10^5 CLL cells were lysed and analyzed by Wb analysis with anti-caspase 3 and anti-PARP antibodies to monitor caspase-dependent apoptosis, and anti-β-actin antibody as a loading control (right panels). **(B)** Freshly isolated CLL cells were cultured as described in (A) and, cell lysates underwent Wb analysis with pS591-SHP-1 antibody. The blots were then stripped and reprobed with anti-SHP-1 antibody. **(C)** Freshly isolated CLL cells were cultured as described in (A). After such treatment, SHP-1 was immunoprecipitated from the cytosol and tested for the basal tyrosine phosphatase phosphatase activity in vitro on [^{32}P]phospho-band 3**.** Data are expressed as mean ± SD from 4 experiments performed in triplicate. Compared with the control, changes due to MP07-66 were statistically significant (*P ≤ 0.01). **(D)** Freshly isolated CLL cells were cultured in the absence (lanes 1 and 5) or presence (lanes 2-4 and 6-8) of 8 µM MP07-66 supplemented without (lanes 1-2 and 5-6) or with 5 nM OA (lanes 3 and 7) or 25 µM PTP I-I (lanes 4 and 8) for 0 and 6 h. Cells were then lysed and analyzed by Wb analysis with anti-pY380-procasp8 and anti- pY307-PP2Ac antibodies. The blots were then stripped and reprobed with antibodies anti-procasp8 and anti-PP2Ac

by annexin V–PI flow cytometry (Figure 26A). Nintedanib proved moderately effective at inducing apoptosis of CLL cells at 6 to 12 h, its efficacy being largely improved by the concomitant presence of MP07-66, which itself exhibited a pro-apoptotic activity overlapping that of nintedanib when used as single agent. Similarly, co-cultures of CLL cells with bone marrow mesenchymal stromal cells were treated as above with overlapping results (data not shown). Moreover, to explore how PP2A activation took part in the apoptotic process induced by MP07-66, CLL cells were treated with 15 µM nintedanib for 6 h in the absence or presence of okadaic acid (OA), a phosphatase inhibitor that is highly selective toward PP2A in the low nanomolar range (Fernandez et al., 2002). As expected, 5 nM OA drastically reduced the apoptotic rate of CLL cells treated with MP07-66 or the combination with nintedanib, but only to a lower extent with nintedanib

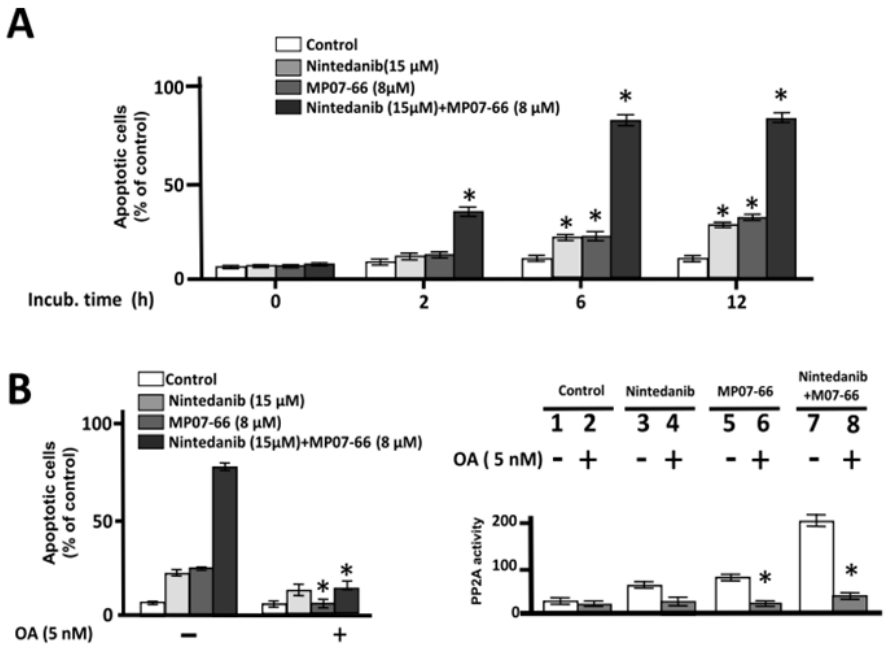

Figure 26: Effect of the combined action of nintedanib and MP07-66 on CLL cell survival. The experiments described below were followed annexin V–PI flow cytometry to assess the effect of the treatments on the vitality of CLL cells. Quadrant analysis after flow cytometry was histogrammed and expressed as mean percentage of early and late apoptosis ± SD from three separate experiments performed in triplicate. **(A)** Freshly isolated CLL cells were cultured in the absence or presence of either 15 µM nintedanib, 8 µM MP07-66, or a combination of 15 µM nintedanib and 8 µM MP07-66 at different time intervals. Compared with the effect of the compounds alone, changes due to the combination of the compounds were statistically significant (*P ≤ 0.01). **(B)** Freshly isolated CLL cells were cultured as described in (A) supplemented without or with 5 nM OA (rigth panels) for 6 h. Compared with the effect of the compounds alone or in combination, changes in the apoptotic rate due to OA were statistically significant (*P ≤ 0.01). The PP2A phosphatase activity performed on the same lysates is shown on the right-hand panel and reported as arbitrary units. Compared with the control, changes of the phosphatase activity due to OA

alone (Figure 26B, left-hand panel). Moreover, the PP2A activity assay performed by using a commercial PP2A assay kit on cell lysates of CLL cells treated as above showed a trend similar to that observed for the apoptotic rate (Figure 26B, right-hand panel). Overall, these data corroborate the hypothesis that the inhibition of PP2A is central to CLL cell viability and that its activation is facilitated by the supportive action of SHP-1, as demonstrated by the effect generated by the simultaneous use of the respective activators.

Discussion

In the present study, we show that nintedanib induces caspase-dependent apoptosis in CLL cells by directly activating the cytosolic pool of the tyrosine phosphatase SHP-1, which in turn dephosphorylates, and thus activates, pro-apoptotic key players such as procaspase 8 (procasp8) and PP2A.

SHP-1 is a tyrosine phosphatase known to negatively regulate signalling in cells of hematopoietic lineage and in B cells modulating the response to antigens and contributing to the development of tolerance to self-antigens. In CLL, SHP-1 undergoes multiple regulatory mechanisms leading to spatial and functional segregation, which is likely to play a relevant role in maintaining the cancer phenotype (Tibaldi et al., 2011). Phosphorylation of different residues in particular, especially at the C-terminus, significantly changes the activation status and localization of SHP-1, phospho-Y536 being typical of the activated pool bound to the plasma membrane co-receptor CD5, and phospho-S591 characterizing the inhibited pool of SHP-1 in the cytosol. This latter form appears to be one of the factors that sustains the aberrant Lyn-dependent tyrosine phosphorylation of countless proteins in the cytosol of CLL cells, which ultimately is key to the anti-apoptotic signalling network of this disease (Contri et al., 2005; Zonta et al., 2014; Zonta et al., 2015). Here, we demonstrate that nintedanib, a small molecule known to act as triple angiokinase inhibitor within the low-nanomolar range (Hilberg et al., 2008), (Roth et al., 2009) and introduced into clinical practice for the management of pulmonary fibrosis and lung adenocarcinoma (McCormack, 2015), activates the cytosolic fraction of SHP-1 by circumventing pS591-dependent inhibition, as recently described in another cancer model (Tai et al., 2014). Significantly, in addition to leaving SHP-1 at the plasma membrane unaffected, as expected, nintedanib only marginally modifies tyrosine kinase activities even at micromolar concentrations in CLL cells. On the other hand, a dramatic drop in tyrosine phosphorylation occurs as a result of SHP-1 activation in the cytosol, and caspase-dependent apoptosis ensues, suggesting that the massive tyrosine

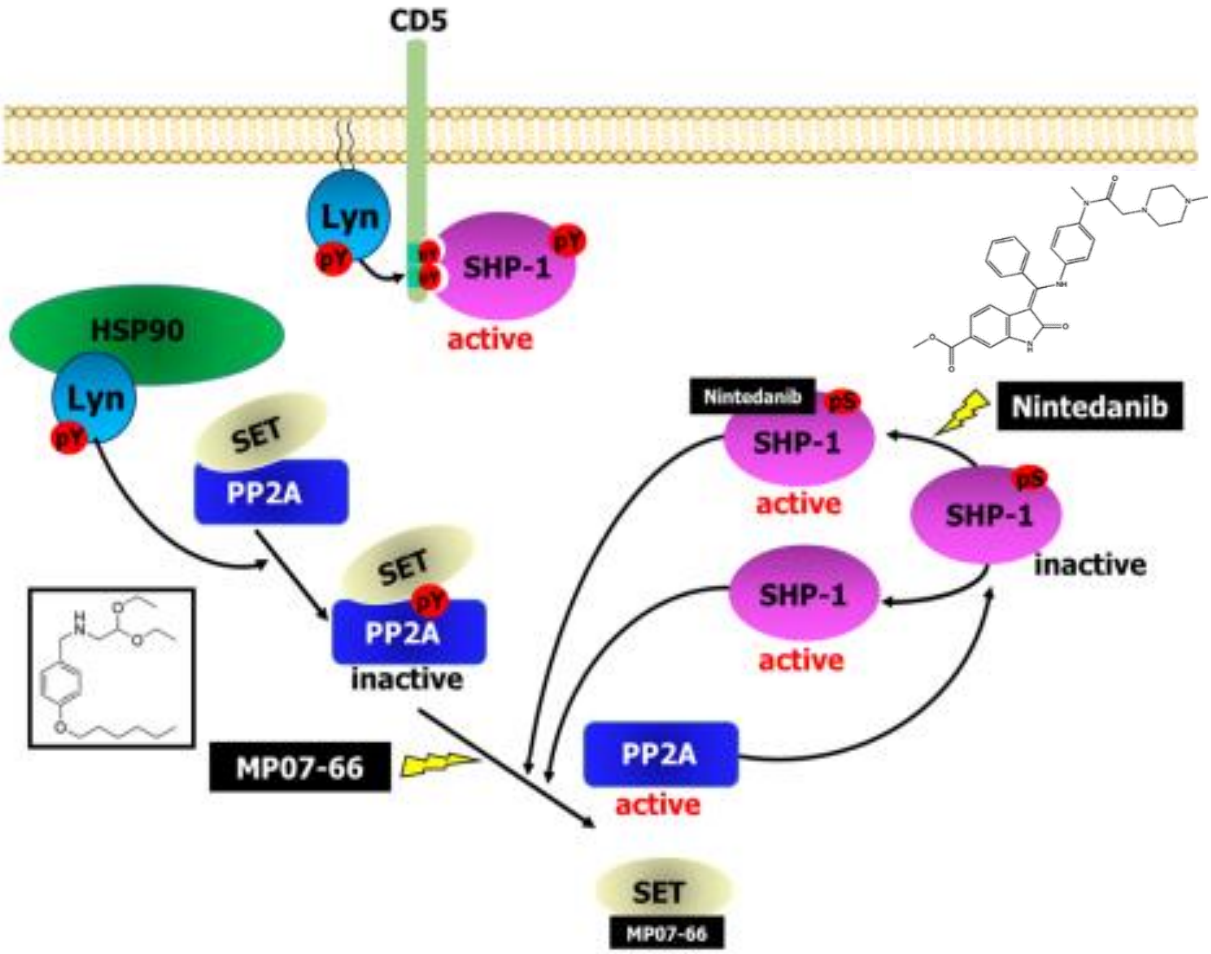

Figure 27. Working model of the positive feed-back signalling loop triggered by the combination of nintedanib and MP07-66 resulting in CLL cell apoptosis. Phosphorylation-dependent inhibition of PP2A by the aberrant form of HSP90-bound Lyn is one of the key mechanisms that take part in the maintenance of the leukemic status of CLL cells. Under these conditions, the inability of PP2A to dephosphorylate and activate the tyrosine phosphatase SHP-1 supports the Lyn-mediated high level of cytosolic tyrosine phosphorylation, which ultimately sustains the oncogenic machinery in CLL cells. By directly activating SHP-1 and PP2A respectively, nintedanib and MP07-66 bring about the dephosphorylation of crucial players in the deranged signalling network of CLL, thus switching off pro-survivals and anti-apoptotic signals. Solid lines with arrows: phosphorylation; dotted lines with arrows: dephosphorylation.

phosphorylation in CLL cells directly impinges on the function of factors that counteract the oncogenic machinery. Notably, although genetic ablation or pharmacologic inhibition of SHP-1 prevent the caspase-dependent apoptosis evoked by nintedanib, again supporting the hypothesis that the action of this drug is mediated by SHP-1, caspase-independent apoptosis was previously observed, which is in line with the role of the CD5-bound form of SHP-1 as a pro-survival agent in CLL (Tibaldi et al., 2011). Our findings clearly indicate that the partitioning of the two forms of SHP-1 is central to their differentiated function, at

the plasma membrane in an active form taking part in a signalosome that orchestrates survival signals, and in the cytosol remaining constrained in an inhibited conformation. We have shown that this condition prevents dephosphorylation of cytosolic Lyn targets with pro-apoptotic potential, as is the case of procasp8 and PP2A, the precursor of an effector caspase and a major serine/threonine phosphatase acting as a tumor suppressor respectively, substantially contributing to the suppression of their activity and ultimately to the survival of CLL cells. Procasp8 occurs as an inactive homodimer in CLL cells, the trigger for dimerization being the phosphorylation of Y380 due to the anomalous cytosolic activity of Lyn (Zonta et al., 2014). Here, nintedanib, via direct activation of cSHP-1, induces dephosphorylation, autocatalysis and activation of procasp8, which explains the caspase-dependent apoptosis observed. As to PP2A, Lyn-mediated phosphorylation at Y307 of the catalytic subunit stabilizes its interaction with its physiologic inhibitor SET, thereby hampering the activity of the phosphatase. This in turn results in the persistent serine/threonine phosphorylation of PP2A substrates, including cSHP-1, and propagates pro-survival and anti-apoptotic signals (Zonta et al., 2015). Conversely, nintedanib-activated cSHP-1 dephosphorylates PP2A, facilitating the disruption of the PP2A/SET complex, with activation of PP2A itself and dephosphorylation of SHP-1 (Figure 26D), triggering the apoptotic response. This latter event is magnified by the combination of nintedanib with MP07-66 in that this latter compound directly interferes with the interaction between PP2A and SET (Zonta et al., 2015), further aiding in the reactivation of PP2A. In this scenario, nintedanib and the FTY720 analogue MP07-66, direct activators of SHP-1 and PP2A respectively, appear to initiate a positive feedback signalling loop which opposes Lyn-mediated oncogenic signalling, thus promoting the dephosphorylation of crucial players in the deranged signalling architecture of CLL, switching off anti-apoptotic signals and unleashing cell death. In conclusion, our findings indicate that phosphatase activators may represent a new weapon in the armoury against this form of leukemia, especially in the light of the heterogeneity and the unavoidable progression of the disease as well as the

resistance to the front-line drugs currently in use, not to mention the adverse effects recently reported for the most promising second-line drugs (Brown, 2015; Barr et al 2016).

MATERIALS AND METHODS

MATERIALS

PARP polyclonal antibody was from Roche Applied Science (Mannheim, Germany). Anti-PP2A- C□/ß, anti-p-PP2A-C□/ß (Tyr307), anti-I2PP2A/SET and anti-caspase 3 antibodies, protein G PLUS-Agarose was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-pS591-SHP1 and anti-pY536 SHP-1 were from ECM Biosciences (Versailles, KY, USA). Anti-procasp8, anti- phosphotyrosine (pY) and anti-SHP-1 antibodies, SHP-1 Inhibitor-I (PTP-I-I), and Ser/Thr Phosphatase Assay Kit 1 (K-R-pT-I-R-R) were from Millipore-Merck (Billerica, MA, USA). Anti- phospho-Tyr380 procasp8 (anti-p-casp8) antibody was from Abcam (Cambridge, UK). Anti-ß-actin antibody and okadaic acid were from Sigma-Aldrich (St Louis, MO, USA). Pierce ECL Western Blotting Substrate, Protease inhibitor cocktail were from Thermo Fisher Scientific (Strasbourg,
France). The SHP-1 siRNA and negative control siRNA were provided by Santa Cruz Biotechnology (Santa Cruz, CA, catalog number sc-29478). [γ^{32}P]ATP was from Perkin Elmer (Milan, Italy). Nintedanib was from Selleckchem (Houston, TX, USA). Caspase-8 Colorimetric Assay Kit was from Alexis Corporation, (Lausen, CH).

METHODS

Ethics Statement

Written informed consents were obtained from all patients, prior to sample collection, according to the Declaration of Helsinki. The ethical approval for our study was obtained from the local ethical committee of "Regione Veneto on Chronic Lymphocytic Leukemia".

Patients, cell separation and culture conditions

B cells from 37 CLL untreated patients were purified and cultured as previously described by our group, and subjected to the treatments described throughout the text. The patients' relevant features are reported in Table 1.

Cell lysis and subcellular fractionation

For total lysates, normal and CLL cells (5×10^5 for each assay) were rapidly lysed in a buffer containing 62 mM Tris/HCl buffer, pH 6.8, containing 5% glycerol, 0.5% SDS, and 0.5% β-mercaptoethanol. ; Trentin et al., 2008). For subcellular fractionation, CLL cells (15×10^6 for each assay) were disrupted and homogenized in isotonic buffer containing 50 mM Tris/HCl, pH 7.5, 0.25 M saccharose, 1 mM orthovanadate, and protease inhibitor cocktail (Boehringer) and centrifuged 10 min at 10,000g in order to separate the particulate fraction containing cell debris, nuclei, and other cellular particles. The supernatant was then subjected to ultracentrifugation for 1 h, performed at 105,000g, to separate cytosol from microsomes (particulate fraction) (Tibaldi et al., 2011). Protein concentration was determined by the Bradford method.

Immunoprecipitation of SHP-1

Cells were disrupted on ice by sonication (three cycles of 5 s at 22 Hz intervalled by 15 s) in isotonic buffer containing 1% NP-40, 20 mM Tris–HCl, pH 7.4, 250 mM Sucrose, 2 mM EGTA, 150 mM NaCl, phosphatase and protease inhibitor cocktails. The lysates were centrifuged at 15 000 *g* for 10 min at 4 °C. The supernatants were immunoprecipitated for 2 h at 4 °C with the anti-SHP-1 antibody and immune complexes were recovered by incubation for 1 h with protein A-Sepharose previously saturated with bovine serum albumine. The immunocomplexes were washed three times in 50 mMTris/HCl (pH 7.5), 0.05% NP-40, protease inhibitor cocktail and submitted to SHP-1 activity assays.

SHP-1 activity assay

SHP-1 activity was tested in SHP-1 immunoprecipitates in the presence of 0.3 μg of [^{32}P]phospho-band 3 as a substrate as described in (Tibaldi et al., 2014). After 10-min incubation at 30°C, the assays were stopped and subjected to SDS-PAGE. The extent of [^{32}P]phospho-band 3 dephosphorylation was evaluated either by

analysis on a Packard Cyclone or by autoradiography and, after excision of the band 3, by liquid scintillation counting.

[32P]-phospho band 3 preparation

Band 3 was phosphorylated by incubating erythrocyte ghosts (15 μg) at 30°C with the tyrosine kinases Syk and Lyn in the presence of [γ^{32}P]ATP as elsewhere described[1]. After 10-min incubation, the sample was centrifuged at 14,000 *g* and the pellet washed 3 times with 25 mM Tris, pH 8.0, 1 mM EDTA, 0.02% NaN_3, 10% glycerol, 10 mM β-mercaptoethanol, 10 mg/ml leupeptin, and 50 mM phenylmethylsulphonyl fluoride and then resuspended in the same buffer for the phosphatase activity assay.

PP2A activity assays

PP2A activity assays from samples undergoing the various treatments utilized throughout the study was measured by using the Malachite Green-based Phosphatase Assay Kit 1(K-R-pT-I-R-R) following the manufacturer's instructions.

Casp8 activity assay

Casp8 activity from samples undergoing the various treatments utilized throughout the study was measured using a Caspase-8 Colorimetric Assay Kit according to the manufacturer's instructions.

Apoptosis assays

CLL cells subjected to the various treatments applied throughout the study were collected forflow cytometric analysis. Ten thousand cells per sample were acquired with the use of BD FACS Diva software (version 7.0), and data were analyzed by plottingon an annexin V–PI logarithmic scattergram.

Western blotting

Whole cell lysates and different cell fractions were run in 10% SDS-PAGE and transferred to nitrocellulose membranes. After 1 hour of treatment with 3% bovine serum albumine at room temperature, membranes were incubated with the appropriate antibodies overnight. Immunodetection was carried out with the ECL Western Blotting Substrate on the Kodak Image Station 4000mm Pro Digital System (Eastman Kodak, Rochester, NY, USA). Membranes, when required, were reprobed with other primary antibodies after stripping with 0.1 M glycine (pH 2.5), 0.5 M NaCl, 0.1% Tween 20, 1% β-mercaptoethanol and 0.1% NaN_3 for 2 x 10 minutes.

Cell transfection

SiRNA transfection into CLL cells was performed using the AMAXA nucleofection system (AMAXA, Cologne, Germany). Briefly, cells ($5x10^6$) were resuspended in 100 µl of Nuclefector Solution, mixed with 300 nM of siRNA duplex (control or against SHP-1), and electroporated using program U-015 on AMAXA nucleofector device. Cells were then transferred to 37°C preheated medium and incubated for 48 h at 37°C, 5% CO_2.

In vitro tyrosine kinase assays

To analyze cellular tyrosine kinase activity, CLL cells (4×10^5 for each assay) were lysed by adding 0.5% Triton X-100 and phosphatase and protease inhibitor cocktails in a 25-µl volume for 15 minutes. The tyrosine kinase activity was then tested in 50 µl of phosphorylation medium containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 30 µM [γ^{32}P]ATP (Amersham Pharmacia Biotech) (specific activity 1,000 cpm/pmol), 200 µM sodium orthovanadate, and either 1 mg/ml random polymer polyGlu4Tyr (Sigma-Aldrich) or 200 µM cdc2(6–20) peptide used as exogenous substrates. After 10 minutes of incubation at 30°C, the reactions were stopped and the samples were loaded on SDS/PAGE. Substrate 32 P-phosphorylation was quantified on a Packard Instant Imager.

Statistical analysis

The data presented as mean ± SD were compared using one-way analysis of variance followed by Bonferroni *post hoc* test or Student's *t*-test. A *P*-value ≤ 0.01 was considered as statistically significant. All statistics were performed using GraphPad Prism (version 5) statistical software (GraphPad Software; San Diego, CA, USA).

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